CHAPTER 5: UTILIZATION OF OCT SETUPS FOR MEASUREMENT OF BIREFRINGENCE AND ELASTIC PROPERTIES OF BREAST TISSUES AND FOR MONITORING GROWTH DYNAMICS OF TUMOR SPHEROIDS.

Abstract: In this chapter we describe the use of OCT for quantitative assessment of the birefringence and elastic properties of resected human breast tissue samples. The estimated values of birefringence obtained from the PSOCT imaging showed that the benign breast tissue samples have significantly higher birefringence as compared to the malignant tissue samples. Further, using OCT based elastography measurements, the stiffness coefficients of these tissue samples were also quantified. A significant difference in the stiffness coefficients of the three breast pathologies, normal, benign and malignant, was observed. The use of OCT to monitor the growth dynamics of tumor spheroid non-invasively is also discussed. Here, the study showed that in comparisons with microscopy (which provides only 2D information) the volume estimates provided by OCT were in much better agreement with the total cell count of tumor spheroids measured using hemocytometer.
5.1 Introduction

It is known that breast cancer is often accompanied by fibrosis [86]. Since collagen fibers are birefringent, it is expected that the neoplastic changes would lead to changes in the polarization parameters which can be monitored by polarization sensitive measurements. Further, since the mechanical properties of breast tissue depend on its structural organization, any alteration in the organization results in significant variation in the mechanical properties of different pathological tissues. These macroscopic differences in the mechanical properties of the breast tissues have been the basis of the conventional approach of manual palpation for the detection of breast tumors. We have investigated the use of the OCT and PSOCT setups for measurements of the birefringence and stiffness coefficients of the breast tissues to discriminate among the normal, malignant and benign tissue sites.

We have also used OCT to monitor growth dynamics of tumour spheroids. Tumor spheroids are three-dimensional cell cultures that mimic the avascular solid tumours. Studies have shown that the tumour spheroids exhibit structural, metabolic, functional and growth patterns similar to that of solid tumours. These are therefore, a good model system for understanding tumor growth and its organization. Tumour spheroids are also being increasingly used for studying response of solid tumours to photodynamic therapy [87, 88, 89].

5.2 Resected human breast tissue samples

Pathologically characterized tissue samples were obtained from the Pathology Department of CHL-Apollo Hospital, Indore. The tissue samples were kept preserved in formalin
(10%) at room temperature (~ 24°C). Tissue samples from only those patients were included in the study for which the histopathological diagnosis was unambiguous. The histo-pathological report provided by the histopathologist was taken as the gold standard for this study. The samples were of three categories: normal fatty adipose tissues, fibroadenoma (benign tissues) and invasive ductal carcinoma (malignant tissues). The normal tissue samples were obtained from uninvolved areas of the resected cancerous and benign specimens based on histopathologist’s assessment.

5.3 Measurement of birefringence of breast cancer tissue using PSOCT

In Figure 5.1 we show the intensity images of breast tissue samples of different pathologies. The intensity and birefringence (or retardation) images of breast cancer tissues were acquired using PSOCT setup discussed in chapter 2 (section 2.6.1). The OCT image of normal tissue, which is primarily composed of large sized lipid-filled adipocytes [90], is shown in Figure 5.1A (i). The spatially periodic highly scattering boundaries seen in the image can be attributed to the large adiposities. In contrast to the low-scattering patterns of adipocytes of normal tissue, the abnormal tissues (malignant Figure 5.1A (ii) & fibroadenoma 5.1A (iii)) show relatively dense and homogeneous scattering which could be due to increased cell density in tumor tissue [90]. Using the intensity image, while it was possible to discriminate abnormal from normal it was difficult to discriminate between malignant and the benign tumor tissue. The retardation images of the same tissue samples in gray scale are show in Figure 5.1B. For normal breast tissue as in the OCT intensity image, the retardation images show the adipocytes boundaries only. However for malignant and benign tumor, in contrast to intensity images the retardation images show
marked differences. The transition from black to white band in benign PSOCT image represents the change in the accumulated phase retardation from 0 to $\pi/2$.

Figure 5.1: (A): Intensity images (i) normal, (ii) malignant, and (iii) benign of resected breast tissue samples. (B): Retardation images (i) normal, (ii) malignant, and (iii) benign of resected breast tissue samples. Image size: 1 mm (depth) × 2 mm (lateral).

Figure 5.2 shows the calculated phase retardation as a function of the depth for malignant and benign samples respectively. The malignant tissue samples display a phase retardation plot increasing at a slower rate in comparison to benign tissue samples, indicating a lower birefringence. The birefringence ($\Delta n$) of the sample can be calculated by a measurement of the slope of the phase retardation profile along the depth using the following expression [91]

$$\Delta n = \text{slope} \times \frac{\lambda}{2\pi}$$  \hspace{1cm} (5.1)

where $\lambda$ is the wavelength of the source used.
Figure 5.2: Phase retardation depth profile of malignant (circle) and benign (square) breast tissue. The linear fitting of the depth profile is shown by solid (benign) and dashed (malignant) lines. The arrow shows tissue top surface.

The mean birefringence value of benign samples averaged over 12 different samples $(4 \pm 1 \times 10^{-4})$ was significantly higher $(p < 0.001, \text{t-test})$ than that of the malignant tissue $(8.0 \pm 3.0 \times 10^{-5})$. These changes can be attributed to the fact that the fibroadenoma has more ordered collagen which contributes to an increased birefringence as compared to the malignant breast tissue [92,93]. The results indicate that the PSOCT can be used as a potential tool for effective discrimination of breast tissue samples.

The feasibility of PSOCT to discriminate the normal, benign and malignant breast tissue samples has significant potential for tissue diagnosis and identification of tumor margins of normal and abnormal tissue. In Figure 5.3 we show a representative image or identifying the tumor margin. The arrows in the image differentiate the normal tissue from the tumor tissue. The image region left side of the arrows shows the cell boundaries of the adipocytes cells while the right region of the image shows the dense scattering of tumor tissue.
Figure 5.3: (a) Intensity and (b) retardation images of breast tissue. Arrows show the tumor margin. Image size: 1 mm (depth) × 2 mm (lateral).

5.4 Measurements of elastic properties of resected human breast tissue samples

Several elastography techniques such as ultrasound elastography, magnetic resonance elastography, etc. have been used to estimate or assess the changes in the mechanical properties of the tissues under compression at a microscopic level. These techniques are based on the principle that by applying a known pressure on the tissue sample and by measuring the induced strain in the sample, information about the mechanical properties (e.g. tissue stiffness) can be extracted. Optical elastography which includes micro-/nano-speckle method (electron speckle photography), diffusing wave spectroscopy (DWS), etc., offers the potential for increased spatial resolution and better strain resolution as compared to ultrasound and magnetic resonance elastography techniques. OCT has also been used for elastographic measurement of the biological tissues. Schmitt [94] reported the measurements of displacements as small as a few micrometers in heterogeneous gelatin phantoms containing scattering particles in addition to human skin as a function of depth.
under a compressive load using OCT-elastography. This technique has also been applied for the investigation of arterial wall biomechanics [95,96], atherosclerotic plaques [97,98], and engineered tissues [99].

For measurement of stiffness coefficients, OCT has been used to image displacements induced within the resected human breast tissue samples subjected to external axial compressive loads. A speckle-tracking technique based on two-dimensional cross correlation of successive OCT images (pre-compressed and post-compressed) was employed for a quantitative measurement of the induced local speckle displacements. Resultant strains at each pixel have been calculated using the two-dimensional displacements maps for normal, benign and malignant breast tissue samples. The stiffness of each sample has been quantified in terms of the modulus of elasticity using the stress-strain relationship. To validate the methodology of OCT elastography measurements including the speckle-tracking method, the overall data reduction approach has been examined by estimating the elastic properties of tissue-mimicking gelatin phantoms. The procedures applied for phantom preparation and OCT elastography measurements are based on the method suggested by Rogowska et al. [98].

Figure 5.4(a) shows a schematic diagram of the loading arrangement employed to compress the tissue samples. The tissue compression unit consists of an L-shaped metallic plate mounted on a stepper motor vertical translation stage. The resolution of the motorized translation stage is 4 µm. Here the metallic plate acts as the loading component. The translation stage traverses in the vertical direction, which in turn moves the metallic plate, and the plate compresses the tissue sample in the axial (downward) direction. The horizontal arm of the L-shaped metallic plate has a clear hole of about 5 mm diameter in the middle to allow the OCT probe beam to pass through. A cover slip placed on the top of
the sample serves as an optical window as well as a compression plate. The objective lens in the sample arm of the OCT setup focuses the light beam on the tissue sample. As the translation stage is lowered, an approximately uniform pressure is applied to the sample, compressing its surface along the axis of the sample beam. Axial load exerted on the sample (after correcting for the weight of the sample holder) is monitored using a load cell kept below the sample holder. The resolution and maximum capacity of the load cell employed in the present work are $4.9 \times 10^{-4}$ N and 4.9 N respectively. In the experiments, the relaxation of load for a particular imposed displacement was found to be quite small ($\leq 5\%$). Furthermore, this variation has been taken care of by using an average of initial and final values of axial compressive loads for the determination of elastic properties of the tissue samples. To monitor the uniformity of the pressure applied on the tissue surface, the displacements of the top layer of the tissue was measured for different values of compressive loads. Across the region of imaging (~2 mm), nearly uniform displacement of the top layer in the downward direction was observed for a given value of compressive load, confirming the uniformity of the pressure applied. This also confirms the establishment of full contact of the metallic plate with top surface of the tissue samples. The response of the breast tissue samples under axial compressive loading is shown in Figure 5.4 (b). The figure shows average displacements of the top surface of normal and malignant samples with respect to the axial load. It can be seen that the tissues exhibit nearly linear mechanical behaviour up to a value of ~ 0.30 mm of the average displacement of the top layer under compression. In the experiments reported in the present work, the maximum displacement of the top surface of the tissue samples (corresponding to the maximum load applied) was about 240 µm. The analysis for the determination of elastic coefficients of tissue samples was carried out within this limit wherein the force-displacement response of the samples is nearly linear.
5.4.1 Phantom preparation

For preparation of tissue phantoms, a mixture of gelatin (800 mg) and agarose (100 mg) was dissolved in 6 ml of boiling water and the solution was continuously stirred to avoid the formation of lumps within the solution. A fixed amount of activated charcoal particles (25 mg) was added in the solution as the scattering centers. The mixture was poured into a Petri dish (40 mm diameter) and refrigerated for several hours. After refrigeration, the phantoms were cut into 20 mm × 20 mm × 4 mm thick squares, covered with a glass slide of thickness ~1.5 mm and were subjected to axial compressive loading using an arrangement schematically shown in Figure 5.4(a). The original and compressed phantoms were scanned with OCT at an axial resolution of 11 µm.
5.4.2 Displacement and strain calculation

The interpretation of OCT images is usually based on the visualization of the two-dimensional, cross-sectional microstructures within the tissue samples. To derive quantitative information from the recorded OCT images, several data processing steps are required. In the present work, a speckle-tracking method based on two-dimensional cross-correlation technique has been employed to derive displacement and strain information from the OCT images of pre-compressed and post-compressed breast tissue samples and tissue-mimicking phantoms.

Cross-correlation is a standard pattern matching approach to identify structures in the initial image and track them in the successive images. For each pair of images, a two-dimensional window (kernel) of dimension $m \times n$ (width $\times$ height) is defined in the initial OCT image (pre-compressed). This window is centered at every pixel in the successive images (post-compressed) and a 2-D array of cross-correlation coefficients is calculated.

Mathematically, the cross-correlation coefficient at a position $(i, j)$ on the image can be expressed as

$$C(i, j) = \frac{\sum_{i=1}^{m} \sum_{j=1}^{n} (\text{kernel}[i + n][j + m] - \overline{\text{kernel}})(\text{image}[i + n][j + m] - \overline{\text{image}})}{\sqrt{\sum_{i=1}^{m} \sum_{j=1}^{n} (\text{kernel}[i + n][j + m] - \overline{\text{kernel}})^2 (\text{image}[i + n][j + m] - \overline{\text{image}})^2}}$$ (5.2)

Here, $\overline{\text{kernel}}$ and $\overline{\text{image}}$ are the mean values of the pixels under an area of dimensions $m \times n$ in the pre-compressed and post-compressed images respectively. The cross correlation coefficient is maximum for the identical regions and displays minima for the uncorrelated regions. Over the entire image, an array of correlation coefficients is calculated and the point of highest cross-correlation magnitude corresponds to the
displacement of pixel \((i, j)\) in the post-compressed OCT image in the axial and lateral dimension. The axial and lateral displacements can be combined to form vectors, which in turn, can be graphically represented as displacement vector maps. In the present work, six different kernel sizes \((11 \times 11, 21 \times 21, 31 \times 31, 41 \times 41, 51 \times 51 \text{ and } 61 \times 61)\) were employed for the gelatin phantoms for comparison and to test the accuracy of the measured displacements. Here the first and second numbers represent the number of pixels in the axial and lateral directions respectively. The optimum kernel size was selected by comparing the mean axial displacement as calculated from the displacement maps for the six kernels and the actual (measured) displacement of the top layer of the phantom under axial compression.

Local strain value \((\varepsilon)\) at a given pixel \((i, j)\) in the image has been calculated from the 2-D axial displacement maps as:

\[
\varepsilon = \frac{d_{i+1,j} - d_{i,j}}{\Delta z}
\]  

(5.3)

Here, \(d_{i,j}\) and \(d_{i+1,j}\) are the displacement magnitudes of two successive pixels in the axial direction under the effect of applied load, \(\Delta z\) being the original distance between these two successive pixels.

5.4.3 Validation experiments with gelatin phantoms

Figure 5.5 shows the OCT images of gelatin phantom subjected to axial compressive load. The pre-compressed image is shown in Figure 5.5 (a) while the displaced OCT images have been displayed in Figure 5.5 (b-f). The phantom-cover slip interface is labelled in Figure 5.5 (a). Compressive load corresponding to the successive displacement (~ 20 µm) as observed in the experiments is about 245 N/m². However, for better visualization of displacement of the top layer of phantoms, images corresponding to alternate load values
(490 N/m²) have been shown in the figure. Bright speckle patterns as seen in the images are due to the presence of the distributed activated charcoal particles embedded in the phantom. A gradual shift of these speckle patterns in the axial direction due to the compressive loading can be seen from the displaced images.

Figure 5.6 show the displacement vector maps for the gelatin phantoms for 31×31, 41×41 and 51×51 kernel sizes. The maps correspond to the images shown in Figure 5.5. A comparison of all the six kernel sizes in terms of accuracy in the measurement of the mean axial displacement has been summarized in Table 5.1. The axial displacement map presented in the figure quantifies the trajectories of the speckle patterns of the phantom and gives a measure of the degree of particle displacement subjected to compressive loading. It is to be seen that with the increasing size of the kernel, the displacement vector maps are less noisy and the vectors are more clearly defined. This transition can be attributed to the fact that the large kernel sizes tend to average out the differences in the displacement of small particles in the phantom. However, larger kernel sizes also limit the ability of the technique to assess the microstructural movement of small speckles within the tissue phantoms. For the selection of the best kernel size, the mean axial displacement has been calculated from the displacement maps of successively displaced OCT images and compared with the actual displacement of the top layer of the phantom. The actual displacement has been measured directly from the recorded OCT images by noting the downward shift in the top layer of the phantom before and after the application of the axial compressive load (245 N/m²). In terms of the percentage error between the calculated and measured displacements, minimum error was observed for 41×41 kernel dimensions (~5%) whereas the errors were seen to be considerably higher for 11×11 (~45%), 21×21 (~30%), 31×31 (~10%), 51×51 (~15%) and 61×61 (~25%) kernel sizes (Table 1). Based on
these results, a kernel size of 41×41 pixels was chosen as the optimum for the cross
correlation technique and also employed for the analysis of resected breast tissue samples.

Figure 5.5: Original (a) and post-compressed (b-f) OCT images of gelatin phantoms. Axial load (in
steps of ~ 245 N/m²) is applied along z-direction to compress the phantoms. For better clarity,
OCT images corresponding to alternate load values have been shown above. The displacement of
the top layer of the phantom (phantom-cover slip interface) in successive images shown above is
about 40 µm.

Table 5.1: Percentage error between calculated mean axial displacement and measured
axial displacement for six different kernel sizes.

<table>
<thead>
<tr>
<th>Kernel size (Pixels)</th>
<th>Calculated mean axial displacement (µm)</th>
<th>Measured axial displacement (µm)</th>
<th>Percentage error</th>
</tr>
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<tbody>
<tr>
<td>11×11</td>
<td>11</td>
<td>20</td>
<td>45</td>
</tr>
<tr>
<td>21×21</td>
<td>14</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>31×31</td>
<td>18</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>41×41</td>
<td>21</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>51×51</td>
<td>23</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>61×61</td>
<td>25</td>
<td>20</td>
<td>25</td>
</tr>
</tbody>
</table>
Figure 5.6: Displacement vector maps for a series of axial compressive loads as obtained for gelatin phantoms by cross correlation technique with three different kernel sizes (31×31, 41×41 and 51×51 pixels). The maps correspond to the OCT images shown in Figure 5.5. (Vertical dimension of each plot (from top to bottom): 1.38, 1.34, 1.30, 1.26 and 1.22 mm. Horizontal dimension: 2.0 mm. Numbers below each row indicate the compressive stress applied in N/m².)
5.4.4 Measurements on breast tissue samples

OCT images of the normal, benign and malignant breast tissue samples were acquired with tissues subjected to compressive loading in the axial direction. The cross correlation technique was used to determine the axial displacement and strain for the breast tissue samples. The elastic properties of normal, benign and malignant tissue samples were calculated using the stress-strain relationship and have been reported in terms of the modulus of elasticity. The present analysis is valid under the assumption that the tissue is incompressible [100,101]. This implies that the Poisson ratio for breast tissue samples is approximately 0.5, and hence, only an elastic modulus is required to characterize the tissue.

Figure 5.7 shows the OCT images of normal, benign and malignant resected breast tissues samples. The pre-compressed images have been shown in Figure 5.7(a) and the successively displaced images due to compressive loading are presented in Figure 5.7(b). The spatially periodic highly scattering boundaries, as seen in the normal breast tissue OCT images (column 1), are primarily due to the presence of large lipid-filled adipocytes cells. Columns 2 and 3 in Figure 5.7 show the OCT images of benign and malignant breast tissues respectively. These images show relatively dense scattering compared to the OCT images of normal samples. The scattering differences between malignant and the benign tissues are not very significant hence these two pathological variations cannot be distinguished based on the qualitative assessment of OCT images alone.
Figure 5.7: Original (a) and post-compressed (b) OCT images of normal, benign and malignant breast tissue samples. The compressive stress is applied along z-direction (axial). Displacement in successive images shown above is about 40 µm.
Figure 5.8: Displacement vector maps for a series of axial compressive loads as obtained for normal, benign and malignant breast tissue samples by cross correlation technique. (Kernel size= 41×41 pixels). (Vertical dimension of each plot (from top to bottom): 1.66, 1.63, 1.59, 1.54 and 1.50 mm. Horizontal dimension: 2.0 mm). The number below each image indicates the compressive stress applied in units of N/m$^2$. )
Figure 5.8 shows the axial displacement vector maps for normal, benign and malignant breast tissue samples. The displacement vector maps for the normal class show a well-defined movement of the speckle patterns in the axial direction. On the other hand, the displacement vectors are not so uniformly distributed for benign, and especially for the malignant samples. For these samples, the vector maps qualitatively highlight the presence of regions where net displacement is either very small or negligible. A comparatively larger variation is also to be seen in the magnitude of axial displacements within a given vector plot for malignant samples. These differences in the distribution of axial displacements of the speckle patterns between the three classes of tissue samples could be attributed to the differences in the elastic properties of the samples as well as to the degree of heterogeneity within a given tissue sample (that was large for the malignant samples considered in the present work).

Quantitative assessment about the relative differences between the elastic properties of normal, benign and malignant breast tissue samples can be made by

Figure 5.9: Stress-strain curves for normal, benign and malignant breast tissue samples.
estimating the modulus of elasticity for these samples. In the present work, the Young’s modulus for each class of breast tissue samples has been estimated by measuring the slope of the respective stress-strain curves. Figure 5.9 shows the stress-strain curves for normal, benign (fibroadenoma) and malignant (invasive ductal carcinoma) breast tissue samples subjected to compressive loading. The stiffness coefficients (Young’s modulus) for each class of tissue samples were estimated by calculating the slope of the respective stress-strain curve using a linear fit. The analysis was performed on 12 samples and the means and standard deviations for each category have been presented. It is to be stated here that for each compressive load, an average of local strain values over all the pixels has been calculated and this value is used for plotting the stress-strain curve for each of the three classes. In quantitative terms, it can be seen that the curve for malignant breast tissue sample is the steepest (slope, a measure of elastic modulus: 16.45 kPa (mean) ± 1.103 (standard deviation)) revealing highest stiffness coefficient among the three classes whereas the curve for benign sample (slope: 9.03 ± 0.215 kPa) falls between the normal (slope: 4.17 ± 0.074 kPa) and malignant. It is to be noted that the values of the standard deviation are relatively low in all the cases, which confirms the repeatability of the experiments and the data reduction approach. These values are also summarized in Table 5.2. The numerical values of Young’s modulus for malignant breast tissue samples are approximately four times higher than the normal tissues whereas for benign tissue samples, it is about 2 times higher than the normal samples. These results are qualitatively consistent with the previous reports on elastic properties of breast tissue by Krouskop [100], Manduca et al. [102], Lorenzen et al. [103] and Samani et al. [104].
Table 5.2: Means and standard deviations of the estimated modulus of elasticity for the three classes of breast tissue samples identified as normal, benign (fibroadenoma), and malignant (invasive ductal carcinoma).

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Benign (Fibroadenoma)</th>
<th>Malignant (Invasive ductal carcinoma)</th>
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<tr>
<td>Elastic modulus (kPa)</td>
<td>4.17 ± 0.074</td>
<td>9.03 ± 0.215</td>
<td>16.45 ± 1.103</td>
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5.5 Imaging tumor spheroids using OCT

Multicellular tumour spheroids are three-dimensional cell cultures that resemble avascular solid tumours [105]. Studies have shown that these spheroids exhibit structural, metabolic, functional and growth pattern similarities to solid tumours [106,107]. Tumour spheroids are therefore a good model systems for studying therapeutic effects of radiation.

For effective testing of drugs being explored for treatment of solid tumours, rapid methods of generation and analysis of spheroids are crucial. For studying the response of tumour spheroids to drugs, the most widely used method is to measure their size and volume by microscopy. However, volume estimation by this method may not be accurate because spheroids formed by different techniques may not be perfect spheres. Histology is also used for studying the response of tumour spheroids to different treatments. This however, is not only laborious and time consuming but is also destructive. Non-invasive techniques that could periodically monitor the size and the shape of spheroids are required for speeding up the process. In the direction, Yu et al. have used holographic optical imaging technique for imaging rat osteogenic sarcoma tumour spheroids [108]. We have
used OCT setup for studying the dynamics of tumour spheroid formation, as it can provide high resolution cross-sectional images non-invasively.

Tumor spheroids generated by modified hanging drop method were used for imaging growth dynamics [109]. Single cell suspension (Human colon adenocarcinoma, colo-205 cells) was prepared by trypsinizing cells grown in monolayers with 0.25% trypsin (w/v) (Himedia). Cell count in the suspension was determined using Hemocytometer and the suspension was diluted in growth medium to have ~ $1.75 \times 10^6$ cells ml$^{-1}$. For generating cellular aggregates, 20µl of the cell suspension having ~35,000 cells was placed as drops on the lids of 60 mm Petri dishes (Tarson, India). The lids with drops were inverted over the dishes containing 2 ml of growth medium and then incubated at 37$^0$ C in 5% (v/v) CO$_2$ incubator. After 24 h of sedimentation, the cellular aggregates were picked up using a pipette. These were transferred to agar coated Petri dishes containing 5 ml of growth medium and allowed to form spheroids. For growing spheroids by the liquid overlay technique, $1 \times 10^6$ cells were plated on the agar coated Petri dishes containing 5ml of growth medium. Clusters of cells could be observed within three to four days of incubation. These were allowed to grow for 7 to 14 days.

The growth dynamics of tumour spheroid formation were imaged using conventional microscopy and OCT imaging setup. In Figure 5.10 (A) we show the OCT images of the spheroids grown for different durations. In all the images, scattering from the agar base is clearly visible. The initially cellular aggregates appear flat with slight curvature towards the edge. With increase in the age of the spheroids, the thickness of the cell layers in the axial direction was observed to increase whereas lateral diameter decreased. In contrast to OCT images, the cellular aggregates and the growing spheroids appeared spherical under microscope (Figure 5.10 B (a-e)). The diameter of each spheroid was estimated by
measuring the geometric mean of the two orthogonal diameters using a calibrated eyepiece reticule (Figure 5.11). The images were taken with a camera mounted on the top of the inverted microscope. Volume of the spheroid images with microscope (50X) was calculated using the formula $V = \frac{4}{3}\pi r^3$ for a sphere where ‘r’ is the radius. The spheroids appeared translucent up to 3 days. Beyond this time point, two distinct regions could be seen in the microscopic images. The central region was dark compared to the outer region. In six-days old spheroids, the area of the dark center increased and diffuse light could be seen only from rim of the spheroids (Figure 5.10B (d)). The observed decrease in the light transmitted through spheroid is due to an increase in thickness. However, actual thickness could not be estimated by microscopic method.

![Figure 5.10: Images of spheroids grown for different durations (a) 0, (b) 4, (c) 5, (d) 6 and (e) 7 days; A) OCT and B) Phase contrast images. Zero day represents time when cell aggregate is transferred from hanging drop to agar coated petridishes. Scale bar: 500 µm](image)

The measurements made by OCT on the axial thickness of spheroids with growth are shown in Figure 5.11. Since OCT images revealed that the tumour spheroids were not spherical, volume was calculated using the formula for oblate spheroid, $V = \frac{4}{3}\pi a^2b$, where ‘a’ and ‘b’ are the radius for the long and short axis respectively. The OCT images provide
the optical depth (physical depth multiplied by refractive index of the medium) hence the physical depth of the spheroid was determined by taking its refractive index to be 1.36 same as for the tissue [110]. Although axial thickness was seen to increase with growth, lateral diameter of spheroids measured by both microscopic and OCT methods reduced with increase in age and were found to be almost constant after 4 days (Figure 5.11). The reduction in lateral diameter may arise because, the non-adherent agar base prevents the cell attachment to surface and therefore even if cell growth expands laterally, layer of cells fold onto itself and grow only in the axial direction [109]. Another reason for the observed decrease in lateral thickness of spheroids may be the increase in cohesiveness of cells in the spheroids. This arises because of an enhanced expression of cadherins and integrin proteins in cells comprising spheroids [111].

![Figure 5.11: Comparison of dimensions of spheroids measured by microscopy and OCT.](image)

Volumes of the spheroid determined by OCT increased with increase in growth and correlated with increase in cell number (Figure 5.12 a). Total number of cells in the spheroids at different time points was evaluated by microscopic counting using hemocytometer (Figure 5.12b). It is interesting to note that for the spheroids prepared by
the method followed by us, although the cell number increased with time, volume determined by microscopy decreased with growth (Figure 5.12a and b). This may be because in this method, a large numbers of cells are used for the hanging drop preparation. This result in formation of large two dimensional cell aggregates and acquires three-dimensional structure on transfer to non-adherent agar by growing only in the axial direction. Since microscopic measurements cannot evaluate the depth accurately, the lateral diameter used for calculation may wrongly estimate a decrease in volume with increase in time of growth. It is also pertinent to note that although the volume measured by OCT method was slightly lower than the microscopic method the difference in the two measurements decreased with an increase in the age of the spheroid. This may be due to a lower axial thickness of the spheroid as compared to its lateral diameter.

![Figure 5.12: (a) Volume of spheroids determined by microscopy and OCT. Inset shows volume of spheroids determined by OCT in expanded scale. (b) Total number of cells in spheroids grown for different days. Cell count was determined using Hemocytometer.](image)

Spheroids generated by the liquid overlay and spinner culture method develop necrotic centers due to the low levels of oxygen and nutrients. With an increase in the age
of spheroids the size of necrotic centers increases [112]. To evaluate the possibility of the use of OCT for studying the morphological changes, we imaged 10 days old spheroids (~200 µm, diameter) prepared by liquid overlay method (Figure 5.13). For identifying the necrotic regions in spheroids, these were stained with 10 µg/ml of propidium iodide (Molecular Probes Inc) for 15 min. These were then examined under a fluorescence microscope (Axiovert 135, Zeiss) using 540 nm band pass excitation and 590 nm long pass emission filters to detect fluorescence from propidium iodide. Spheroids, which showed fluorescence were used for imaging by OCT. The OCT images showed distinct high intensity scattering in some regions. Since these spheroids also showed propidium iodide fluorescence, the high scattering regions can be considered to be necrotic.

Figure 5.13: OCT cross sectional images of the spheroid grown by liquid over-lay method (a-b), a & b are OCT images of same spheroid acquired twice to show the reproducibility of high scattering regions, fluorescence image of a typical spheroid grown for 10 days time stained with propidium iodide. Scale bar: 50 µm (c). Spheroids were stained with propidium iodide (10 µg/ml) for 15 min. The fluorescence was viewed under microscope using 540 nm band pass excitation and 590 nm long pass emission filters respectively. Scale bar: 40 µm.

5.6 Summary

We have carried out OCT and PS-OCT imaging of resected human breast tissue samples belonging to different pathologies. The measured values of birefringence show that the benign (fibroadenoma) breast tissue samples have significantly higher birefringence than
that in malignant (invasive ductal carcinoma) tissue samples. Similarly, the stiffness 
coefficients of these tissue samples were quantified in terms of the elastic modulus 
estimated using the measured displacement vector and axial strain. The results of the 
experiments performed on resected breast tissue samples reveal that the stiffness 
coefficient for benign samples, as calculated from the stress-strain relationship, is found to 
be about 2 times higher than the normal samples, whereas for malignant samples, it is 
approximately 4 times. These results indicate that PSOCT imaging and OCT based 
elastography can be used as the potential tool for effective discrimination of breast tissue 
samples.

We have also used OCT to monitor the growth dynamics of tumour spheroid 
formation. Volume of spheroids estimated by OCT correlated well with the increase in cell 
number as a function of the growth. OCT images also revealed heterogeneous structures in 
the 200 µm spheroids that correlated with necrotic regions observed by the fluorescence of 
propidium iodide. The use of OCT imaging for monitoring the growth of spheroids may 
prove to be useful for evaluating the drug response on tumour spheroids. Compared to 
histology, this technique provides a non-invasive and rapid method for evaluating tissue 
changes.