EXAMINATION OF DIAGNOSTIC FEATURES IN MULTIPHOTON MICROSCOPY 
AND OPTICAL COHERENCE TOMOGRAPHY IMAGES OF OVARIAN 
TUMORIGENESIS IN A MOUSE MODEL 

by 

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SIGNED: Jennifer M. Watson
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DEDICATION

To Mom, Dad, Jeff and Clay. Your love and encouragement have nurtured me.
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... 10
LIST OF TABLES ............................................................................................................ 14
ABSTRACT ...................................................................................................................... 15
INTRODUCTION ............................................................................................................. 17

1.1 Ovarian Cancer ...................................................................................................... 17
  1.1.1 Prevalence & Mortality .................................................................................... 17
  1.1.2 Risk and Risk Reduction ................................................................................. 17
  1.1.3 Detection ......................................................................................................... 19
  1.1.4 Treatment ....................................................................................................... 20
  1.1.5 Types of Ovarian Tumors .............................................................................. 20
  1.1.6 Ovarian Cancer Models .................................................................................. 22
    1.1.6.1 *In vitro* models ..................................................................................... 23
    1.1.6.2 *In vivo* models .................................................................................... 23

1.2 Medical Imaging ................................................................................................... 27
  1.2.1 X-rays and Computed Tomography ............................................................... 28
  1.2.2 Nuclear Medical Imaging .............................................................................. 29
  Positron Emission Tomography .............................................................................. 31
  1.2.4 Magnetic Resonance Imaging ...................................................................... 32
  1.2.5 Ultrasound ................................................................................................... 34
  1.2.6 Optical Imaging ............................................................................................ 35
1.2.6.1 Optical Coherence Tomography ................................................................. 36
1.2.6.2 Photoacoustic Imaging ............................................................................. 42
1.2.6.3 Fluorescence Imaging ............................................................................. 43
1.2.6.4 Confocal Microscopy ............................................................................. 44
1.2.6.5 Multiphoton Microscopy ......................................................................... 45
1.2.7 Contrast Agents for Medical Imaging .......................................................... 51

1.3 Explanation of Dissertation Format .................................................................. 54

PRESENT STUDY ........................................................................................................ 55

2.1 Motivation ........................................................................................................... 55

2.2 Aim 1: Use second harmonic imaging to quantitatively examine the morphological differences in collagen structure in normal mouse ovarian tissue and mouse ovarian tumors. .......................................................................................................................... 56

2.3 Aim 2: Examine the differences in endogenous two-photon fluorescence in normal mouse ovarian tissue and mouse ovarian tumors ........................................................................................................... 57

2.4 Aim 3: Identify changes in ovarian microstructure resulting from disease development by imaging animals in vivo at three time points during a long-term survival study. ....... 59

2.5 Conclusion ......................................................................................................... 61

REFERENCES ............................................................................................................ 62

APPENDIX A: ANALYSIS OF SECOND HARMONIC GENERATION MICROSCOPY IN A MOUSE MODEL OF OVARIAN CARCINOMA ................................................................. 71
A.1 Published Manuscript: Analysis of Second Harmonic Generation Microscopy in a Mouse Model of Ovarian Carcinoma ................................................................. 71
A.2 File Locations ........................................................................................................... 105

APPENDIX B: TWO-PHOTON EXCITED FLUORESCENCE IMAGING OF ENDOGENOUS CONTRAST IN A MOUSE MODEL OF OVARIAN CANCER ...... 107

B.1 Published Manuscript: Two-photon excited fluorescence imaging of endogenous contrast in a mouse model of ovarian cancer ................................................................ 107
B.2 File Locations ........................................................................................................... 145
B.3 Standard Operating Procedure for Sudan Black B stain for staining Lipids ........... 146
B.4 Standard operating procedure for Ziehl-Neelsen modified to stain lipofuscin ........ 148
B.5 Standard operating procedure for IHC staining for F4/80 macrophages .............. 150

APPENDIX C: IN VIVO TIME-SERIAL MULTI-MODALITY OPTICAL IMAGING IN A MOUSE MODEL OF OVARIAN TUMORIGENESIS ............................................ 153

C.1 Manuscript: In vivo time-serial multi-modality optical imaging in a mouse model of ovarian tumorigenesis ....................................................................................... 153
C.2 File Locations ........................................................................................................... 197
C.3 Standard operating procedure for the multiphoton microscope ......................... 198
C.4 Standard operating procedure for the SS-OCT1050 .............................................. 204
C.5 Standard operating procedure for co-localization of SS-OCT and MPM ............. 207
C.6 Standard operating procedure for Ketamine/Xylazine anesthetic for IP injection... 210
C.7 Standard operating procedure for rodent surgery............................... 214
C.8 Standard operating procedure for in vivo mouse ovary imaging with the SS-OCT and MPM ................................................................. 220
C.9 VPR Approval #1 for Animal Subjects .................................................. 227
C.10 VPR Approval #2 for Animal Subjects ................................................ 228
C.11 VPR Approval #3 for Animal Subjects ................................................ 229

APPENDIX D: COMMERCIALIZATION POTENTIAL OF AN OCT-MPM IMAGING SYSTEM FOR OVARIAN CANCER SCREENING ........................................ 230

D.1 Commercialization Potential of an OCT-MPM Imaging System for Ovarian Cancer Screening ................................................................. 230

Clinical Need ......................................................................................... 231
Risk/benefit and Adoption ................................................................. 233
Market Size ......................................................................................... 235
Current State/Competitive comparison .............................................. 236
Technology: Advantages and Challenges .............................................. 237
Barriers to Entry .................................................................................. 241
Reimbursement Strategy ................................................................. 242
Regulatory Approval ........................................................................... 245
Financials ......................................................................................... 247
Summary .............................................................................................. 249
LIST OF FIGURES

Fig. A.1 Images from three ovaries of each diagnosis: normal (A-C), DMBA-effect (D-F), tubular adenoma (G-I), tubular adenoma with dysplasia (J-L), and carcinoma (M-O) ................................................................. 101

Fig. A.2 Average parameter values for parameters used to separate carcinoma from normal and carcinoma from non-carcinoma. TA=tubular adenoma. TD=tubular adenoma with dysplasia ................................................................. 102

Fig. A.3 Average parameter values for parameters used to separate tubular adenoma (TA) from tubular adenoma with dysplasia (TD) ................................................................. 102

Fig. A.4 Plot of power in the high frequency region versus GLCM energy for carcinoma and normal diagnoses ................................................................. 103

Fig. A.5 Plot of contrast with 17 pixel separation vs. contrast with 25 pixel separation for tubular adenoma (TA) and tubular adenoma with dysplasia (TD) ......................... 103

Fig. B.1. (A) TPEF image showing dim fluorescence from a corpus luteum and granulosa cells surrounding a follicle in normal ovary, (B) H&E of normal ovary showing a corpus luteum and a follicle surrounded by granulosa cells ........................................ 134

Fig. B.2. (A) TPEF image showing dim fluorescence from adipose cells and carcinoma cells, (B) H&E of carcinoma specimen with adipose cells and carcinoma cells ........ 134

Fig. B.3. (A) TPEF image showing dim linear bands of fluorescence in a carcinoma specimen, (B) H&E showing collagen abundance in a carcinoma specimen .............. 135

Fig. B.4. (A) TPEF image showing collagen & elastin fluorescence from a blood vessel in a normal ovary. (B) H&E showing blood vessels in normal ovary ................. 135

Fig. B.5. (A) TPEF image showing bright punctate and bright cellular fluorescence in normal ovary, (B) H&E showing foamy cells in normal ovary ......................... 136
LIST OF FIGURES - Continued

Fig. B.6. F4/80 positive macrophages stained brown in (A) normal ovary and (B) carcinoma. Corresponding H&E sections for (C) normal ovary and (D) carcinoma…..137

Fig. B.7. Normal ovary (A) Foamy appearing cells are present in H&E stained section (B) Lipid-filled cells and granules appear black in Sudan Black B stained section (C) lipofuscin appears purple in carbol fuchsin stained section…………………………138

Fig. B.8. Fluorescence imaging shows co-localization of fluorescence and Sudan Black B stained lipid in normal ovary. (A) Unstained ovary section under green light, (B) ovary section stained with Sudan Black B (bright field image), (C) Sudan Black B stained ovary section under green light……………………………………………………………………138

Fig. B.9. Fluorescence imaging shows co-localization of fluorescence and carbol fuchsin stained lipofuscin in normal ovary. (A) Unstained ovary section under green light, (B) ovary section stained with carbol fuchsin (bright field image), (C) Carbol fuchsin stained ovary section under green light…………………………………………………………139

Fig. B.10. TPEF images of normal ovary showing increase in lipofuscin fluorescence with age. (A) 11 months old, (B) 13 months old, (C) 15 months old…………………..139

Fig. B.11. TPEF analysis results for normal ovary at each age……………………………140

Fig. B.12. Lipofuscin (in red/purple) accumulation increases with age. Normal ovary at (A) 11 months, (B) 13 months (C) 15 months of age……………………………………140

Fig. B.13. Representative aged-matched TPEF images for each diagnosis. (A) Normal (B) DMBA effect, (C) tubular adenoma, (D) tubular adenoma with dysplasia, (E) sex-cord tumor, (F) carcinoma………………………………………………………………………………141

Fig. B.14. TPEF particle analysis results for each diagnosis…………………………………142

Fig. B.15. Lipofuscin (stained red/purple) accumulates in cells and granules throughout the tissue of each diagnosis. (A) Normal (B) DMBA effect, (C) tubular adenoma, (D) tubular adenoma with dysplasia, (E) sex-cord tumor, (F) carcinoma……………………………………143
LIST OF FIGURES - Continued

Fig. C.1. H&E stained sections for each diagnosis. A) Normal cycling, B) atrophic, C) tubular hyperplasia, D) cystic tumor, E) fibrosarcoma, F) granulosa-cell tumor......165

Fig. C.2. Normal ovary (CON/CON) OCT images. A) cross-section, B) en face section..............................................................167

Fig. C.3. Normal ovary (CON/CON) MPM images. A) TPEF from surface of the ovary. B) TPEF image from deeper in the ovary. C) SHG from the surface of the ovary. D) SHG from deeper in ovary..............................................................169

Fig. C.4. Normal ovary (CON/CON) time serial OCT images. A) 6 months, B) 11 months, and C) 13 months of age..............................................................170

Fig. C.5. Normal ovary (CON/CON) time serial MPM maximum intensity projection images. A-C) TPEF images. D-F) SHG images..............................................................172

Fig. C.6. Atrophy (CON/DMBA) development in time serial OCT images. A) 6 months (before DMBA dosing) and at B) 13 months of age (after DMBA dosing).........173

Fig. C.7. Atrophy (CON/DMBA) development in time serial MPM maximum intensity projection images. A-B) TPEF. C-D) SHG. A & C are from first time point (before DMBA dosing) and B & D are from third time point (after DMBA dosing).............174

Fig. C.8. Tubular hyperplasia (VCD/CON) development in time serial OCT images. A) 6 months, B) 11 months and C) 13 months of age..............................................................176

Fig. C.9. Tubular hyperplasia (VCD/CON) development in time serial MPM maximum intensity projection images. A-C) TPEF. D-F) SHG..............................................................177

Fig. C.10. Cystic tumor (VCD/DMBA) development in time serial OCT images. A) 6 months, B) 11 months of age..............................................................178

Fig. C.11. Cystic tumor (VCD/DMBA) development in time serial MPM maximum intensity projection images. A-B) TPEF. C-D) SHG..............................................................180
LIST OF FIGURES - Continued

Fig. C.12. Fibrosarcoma (CON/DMBA) development in time serial OCT images. A) 6 months (before DMBA dosing), B) 13 months of age (after DMBA dosing)…………181

Fig. C.13. Fibrosarcoma (CON/DMBA) time serial development in MPM maximum intensity projection images. A-B) TPEF. C-D) SHG…………………………………………………183

Fig. C.14. Granulosa cell tumor (VCD/DMBA) development in time serial OCT. A) 6 months, B) 11 months of age…………………………………………………………………184

Fig. C.15. Granulosa cell tumor (VCD/DMBA) development in time serial MPM maximum intensity projection images. A-B) TPEF. C-D) SHG…………………………………186
LIST OF TABLES

Table 1.1 Percentage of ovarian tumor & malignancy types and their subtypes.............21

Table 1.2 Summary of Medical Imaging Techniques.................................................53

Table A.1 Testing results for 100 iterations (standard deviation in parentheses).
TA=tubular adenoma. TD=tubular adenoma with dysplasia........................................104

Table A.2. Testing results for carcinoma versus normal (standard deviation in parentheses)..........................................................................................................................104

Table B.1. Number of ovaries and images with each diagnosis at each age. Number of
images shown in parentheses........................................................................................144

Table C.1 Number of ovaries with each diagnosis in each dosing group.................163

Table C.2. Summary of characteristics of each diagnosis seen by each imaging
modality..............................................................................................................................187

Table C.3. Ketamine/Xylazine dose (and half-dose) listed by mouse weight.............211

Table D.1. OCT-MPM probe size and flexibility requirements....................................239

Table D.2. Benefits and pitfalls of OCT and MPM probes for clinical use..............241

Table D.3. Comparison of current medical procedures for ovarian cancer.............245

Table D.4. Estimated cost components for device approval (5-year clinical study with 100
patients)........................................................................................................................246

Table D.5. Estimated global revenues from imaging devices in 2012......................248
ABSTRACT

Ovarian cancer is a deadly disease owing to the non-specific symptoms and suspected rapid progression, leading to frequent late stage detection and poor prognosis. Medical imaging methods such as CT, MRI and ultrasound as well as serum testing for cancer markers have had extremely poor performance for early disease detection. Due to the poor performance of available screening methods, and the impracticality and ineffectiveness of taking tissue biopsies from the ovary, women at high risk for developing ovarian cancer are often advised to undergo prophylactic salpingo-oophorectomy. This surgery results in many side effects and is most often unnecessary since only a fraction of high risk women go on to develop ovarian cancer. Better understanding of the early development of ovarian cancer and characterization of morphological changes associated with early disease could lead to the development of an effective screening test for women at high risk.

Optical imaging methods including optical coherence tomography (OCT) and multiphoton microscopy (MPM) are excellent tools for studying disease progression owing to the high resolution and depth sectioning capabilities. Further, these techniques are excellent for optical biopsy because they can image in situ non-destructively. In the studies described in this dissertation OCT and MPM are used to identify cellular and tissue morphological changes associated with early tumor development in a mouse model of ovarian cancer. This work is organized into three specific aims. The first aim is to use the images from the MPM phenomenon of second harmonic generation to quantitatively
examine the morphological differences in collagen structure in normal mouse ovarian tissue and mouse ovarian tumors. The second aim is to examine the differences in endogenous two-photon excited fluorescence in normal mouse ovarian tissue and mouse ovarian tumors. The third and final aim is to identify changes in ovarian microstructure resulting from early disease development by imaging animals *in vivo* at three time points during a long-term survival study.
INTRODUCTION

1.1 Ovarian Cancer

1.1.1 Prevalence & Mortality

Ovarian cancer is the fifth leading cause of cancer deaths in women in the United States, after lung, breast, colorectal and pancreatic cancers. In 2012, there were more than 22,000 new cases and 15,500 deaths due to ovarian cancer (American Cancer Society 2012). Five year survival rates for ovarian cancer are 92% when found local, 72% when cancer has regional spread and 27% for distant spread, giving an overall five-year survival rate of 44% (American Cancer Society 2012). The overall mortality rate is high because mortality rate increases with disease stage and the majority of cases are found at late stages.

1.1.2 Risk and Risk Reduction

As with most cancers, age is a risk factor for ovarian cancer. The majority of deaths due to ovarian cancer occur in women who are 55 and older. There are also a number of lifestyle choices that can affect the risk of developing ovarian cancer. The risk of ovarian cancer increases for women who have never been pregnant, are obese and smoke tobacco (American Cancer Society 2012, Olsen 2007). Lifestyle choices that decrease the risk of developing ovarian cancer include pregnancy (especially early in life) and taking oral contraceptives. Further, the more time spent in anovulation (multiple pregnancies, breast feeding, long-term oral contraceptives), the greater the risk reduction (Ozols 2003,
Cramer 1983). Some medical procedures such as tubal ligation, hysterectomy and salpingo-oophorectomy (removal of the ovaries and fallopian tubes) can also greatly reduce the risk (American Cancer Society 2012, Hankinson 1993). Salpingo-oophorectomy is a frequent choice for high-risk women and reduces risk by 90% (Collaborative Group 2008).

Genetics play an important role in the development of ovarian cancer. Risk is considered moderate for women with a family history of cancer and high for women with known genetic mutations linked to ovarian cancer. Ovarian cancers that are autosomal dominant frequently result in earlier age of onset and account for 5-10% of ovarian cancers (Ozols 2003). Two common gene defects resulting in higher risk for ovarian cancer are the BRCA1 and BRCA2 mutations. Approximately 1 in 800 women have a BRCA mutation (Ozols 2003). BRCA1 mutations result in a 39-46% chance of developing ovarian cancer and BRCA2 mutations result in an 11-23% chance of developing ovarian cancer (Risch 2006, Chen 2006).

Frequently also related to genetics, the existence of other medical conditions in oneself or family members can increase the risk of ovarian cancer (Cramer 1983). Some of these conditions include other cancers (breast, colon, prostate), pelvic inflammatory disease, Lynch syndrome and hereditary non-polyposis colorectal cancer (HNPCC) syndrome (American Cancer Society 2012, Ozols 2003). Also influenced by both genetics and environment, women who have had early menarche and/or late menopause are at an increased risk for developing ovarian cancer (American Cancer Society 2012).
1.1.3 Detection

One of the reasons ovarian cancer is difficult to detect at an early stage is that the symptoms are non-specific to the disease. Symptoms are primarily digestive or gynecologic in nature. Digestive symptoms may include bloating, difficulty eating, feeling full quickly, lack of appetite, constipation, diarrhea, gas, nausea, vomiting, and pelvic or abdominal pain. Gynecologic symptoms can include abnormal menstrual cycles, vaginal bleeding, urinary urgency or frequency and pain during intercourse. Other symptoms can include back pain, weight gain or loss and fatigue (Hennessy 2009).

Detection of ovarian cancer is usually based on a combination of symptoms, family history, imaging and blood tests. Family history can be an indicator of disease because the genetic mutations resulting in ovarian cancer are frequently passed down to offspring. The genetic factors were discussed in more detail in the ovarian cancer risk section. The most common imaging method used to detect ovarian tumors is ultrasound. Ultrasound is good at detecting ovarian masses but results in many false positives, as the majority of ovarian abnormalities found on ultrasound are not cancer. Additional information on clinical and pre-clinical imaging methods for detecting ovarian cancer is described in the medical imaging section.

When ovarian masses are found, blood tests, such as the CA-125, are frequently performed to assess the malignant potential of the mass. Although CA-125 is elevated in many ovarian cancers, like ultrasound, CA-125 testing results in many false positives, as the majority of elevated CA-125 serum tests are due to conditions other than ovarian
cancer. Furthermore, a randomized control trial of over 78,000 women showed that using CA-125 and ultrasound for ovarian cancer screening did not reduce mortality (Buys 2005, 2011).

Other serum markers that have had limited success include HE4, mesothelin, CEA and VCAM1 (Yurkovetsky 2010, Moore 2009, Skates 2001, Anderson 2012). With all serum markers it is best to have a baseline (healthy) value for each individual and track the values over time to detect elevations. However, it is important to keep in mind that even with tracking over time, these serum markers have not been found as specific for ovarian cancer and elevations can be caused by a change in living habits or other medical conditions.

1.1.4 Treatment

Treatment for ovarian cancer usually includes surgery and chemotherapy. Surgery usually includes bilateral salpingo-oophorectomy (removal of the ovaries and fallopian tubes) and hysterectomy (removal of the uterus) (American Cancer Society 2012). In cases where cancer has spread from the ovary, surgery may include surgical debulking including removal of omentum, lymph nodes and abdominal metastases (American Cancer Society 2012, Hennessy 2009). Following surgery, chemotherapy is used to treat remaining disease or if the cancer returns. After treatment, women under 65 are twice as likely to survive as those over 65 years of age (American Cancer Society 2012).

1.1.5 Types of Ovarian Tumors
The most common ovarian tumors are classified into three types: surface epithelial-stromal, sex cord-stromal and germ-cell. Surface epithelial-stromal, sex-cord stromal and germ cell tumors account for 90%, 7% and 3-7% of malignant ovarian tumors, respectively (Chen 2003). The subtypes of the three main types of ovarian tumors are shown in table 1.1. The most common ovarian malignancy is surface epithelial-stromal of the serous subtype, accounting for 50% of all ovarian malignancies (Chen 2003). Ovarian surface epithelial-stromal cancers usually have solid and cystic components. Signs of malignancy include thick septae between cysts, mural nodules, papillary projections and areas of necrosis.

Table 1.1 Percentage of ovarian tumor & malignancy types and their subtypes.

<table>
<thead>
<tr>
<th>Type</th>
<th>% of all ovarian tumors</th>
<th>% of all ovarian malignancies</th>
<th>Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface epithelial-stromal</td>
<td>60</td>
<td>90</td>
<td>Serous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mucinous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Endometrioid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Clear cell</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Transitional cell</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Squamous cell</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mixed epithelial</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Undifferentiated</td>
</tr>
<tr>
<td>Sex cord-stromal</td>
<td>8</td>
<td>7</td>
<td>Granulosa cell</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sertoli-stromal cell</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sex cord with annular tubules</td>
</tr>
</tbody>
</table>
The term “ovarian cancer” is sometimes used to describe any ovarian malignancy, but generally refers to a malignancy of surface epithelial-stromal cell origin. The ovarian epithelial cells are multipotent (can differentiate into multiple epithelial types including endometrial, tubal, intestinal, etc.) lending to the variety of tumor types and the difficulty of finding the cell origin in some cancers (Ozols 2003). Traditional thought is that ovarian cancers develop from “incessant ovulation,” in which repeated ovulatory cycles resulting in high levels of hormones and trauma to the surface epithelium result in malignant transformation (Fathalla 1971). However, the multipotency of epithelial cells also led to the discovery that ovarian cancers can arise not only from the ovarian epithelium but also from the epithelium of the peritoneal lining or the fallopian tubes (Crum 2007, Kurman 2010).

1.1.6 Ovarian Cancer Models

Due to a variety of factors, including the poor understanding of ovarian cancer development, frequent late stage diagnosis and invasiveness of potential early detection
methods and treatments, it is useful to study ovarian cancer models to obtain detailed information before attempting to change clinical practices.

1.1.6.1 In vitro models

In vitro models are cell culture models that allow investigation of specific cell types. These models can be used to investigate single cell types in isolation or in complex systems. In vitro investigation of single cell types includes cell culture where cancer cells are taken directly from the organism or where healthy cells are taken and manipulated to transform them into cancer cells (Adams 1981, Leung 2001, Ausperg 1999, Hofman 1993). These methods are useful for observing cancer cell behavior in a controlled environment. Additionally, in vitro models have been used to determine some genetic mutations that can cause the transformation of human ovarian surface epithelial cells into malignant cells (Liu 2004, Shan 2009, Fleskin 2003).

More complex cell culture systems include co-cultures, where two or more cell-lines are cultured together to see how they interact (Langdon 2001). These co-culture systems include 3D constructs that simulate the stroma and epithelium of human ovaries and surrounding tissues (Zietarska 2007, Kenny 2007).

1.1.6.2 In vivo models

Although in vitro models provide abundant useful information in studying mechanisms for cell transformation, tumor growth and treatment efficacy, in vitro models cannot completely match the complex and highly diverse environment that tumors develop in
within the body. For this reason, *in vivo* models are extremely useful. *In vivo* models used to study ovarian development and ovarian cancer have included fruit flies, rats, mice, guinea pigs, chickens, rabbits and sheep (Naora 2005, Hoyer 2004, 2009, Orsulic 2002, Fredricksen 1897, Vanderhyden 2003). Animal models have the advantage of providing a complete tumor environment (unlike *in vitro*) while still having the ability to genetically and environmentally control the experiment (unlike in humans).

Spontaneous

The best animal models are those that develop ovarian cancer spontaneously, as these models are analogous to ovarian cancer development in women. Hens and mice have been known to spontaneously develop a variety of ovarian tumor types, including adenocarcinoma (Fredricksen 1987). However, like in women, the tumors are not common and do not develop until old age (2+ years for hens and mice) (Vanderhyden 2003). Thus, the extended length of time and quantity of animals needed to study ovarian cancer development in these models makes study of ovarian cancer impracticable.

Recently much work has been done exploring transgenics to cause spontaneous transformation of the ovarian epithelium, leading to ovarian tumor development *in situ*. One method is to inject adenoviruses under the ovarian bursa in mice to alter gene expression, simulating the mutations that result in humans. Using this method, one model cancer was developed by injecting a recombinant adenovirus expressing Cre into double transgenic mice with mutated p53 and Rb tumor suppressor genes (Vanderhyden 2003). A stable transgenic model of spontaneous cancer development is the Fox Chase TgMISIIR-
Tag mouse, developed by Connolly and colleagues (Connolly 2003). These mice express SV40 Tag under control of the Mullerian inhibitory substance type II receptor (MISIIIR) gene promoter (targeted to the epithelium of the reproductive organs). This model develops bilateral undifferentiated ovarian carcinoma with metastatic spread by 3 months of age. By crossing these mice to C57Bl/6 mice for many generations a stable transgenic line was developed such that by breeding TgMISIIR-Tag-DR26 males with wild type C57Bl/6 females, all transgene female offspring develop ovarian cancer (Connolly 2003).

Xenografts

One common method of in vivo cancer study uses xenografts: implantation of ovarian cancer cells into immunodeficient animals. The xenografts are implanted subcutaneously, intraperitoneally or under the ovarian bursa (listed in decreasing order of the respective ease of implantation and increasing order of accuracy to real tumor location). The xenografts come from primary culture of human cancers, or transformed ovarian epithelial cells from humans or animals. The transformation processes have included using murine sarcoma virus (in rat cells), SV40 T antigen transfection (in human and rat cells) and RCAS retroviral vector (in mice) (Shan 2009). Owing to the fact that cancer cells are implanted and not transformed in situ, xenografts are primarily useful for studying metastatic potential of injected cells rather than early ovarian cancer development.

Chemical Induced
Another common *in vivo* method is chemical induced ovarian cancer. These models have an advantage of heterogeneity of tumor types, as is seen in humans. Additionally, tumors develop without the implantation of cancer cells. The disadvantage of this type is that chemoinduced models do not accurately reflect the pathogenesis for ovarian cancer development in humans, as the number of human ovarian cancers caused by environmental toxins are low. Despite this disadvantage, chemoinduced models can provide similar environment and cell and tissue level changes that lead to ovarian cancer. There are a number of chemicals that have been shown to cause ovarian tumors in mice (Maronpot 1987, Collins 1987, Craig 2010, Hoyer 2004, Hoyer 2009). 1,3-Butadiene causes benign and malignant granulosa cell tumors (Hoyer 2004). Nitrofurazone causes benign mixed tumors and granulosa cell tumors (Hoyer 2004). Vinylcyclohexene diepoxide combined with dimethylbenzanthracene has caused a multitude of different tumor types in rats and mice including Sertoli-Leydig cell tumors, granulosa cell tumors, tubular adenomas and adenocarcinomas (Hoyer 2009, Craig 2010, Marion 2012). The ability to develop adenocarcinoma is extremely valuable in an *in vivo* model since adenocarcinoma is the most common malignancy in human ovaries.

Another reason for using chemical induced models is to simulate menopause. Mice usually continue ovulating their entire life and do not go through menopause mid-life like humans. In order to create an acyclic, menopause-like state, a number of ovotoxic chemicals can be used to kill the ovarian follicles including chemotherapeutic agents (CPA), polycyclic aromatic hydrocarbons (dimethylbenzanthracene, benzopyrene, methylcholanthrene), occupational chemicals (butadiene, vinylcyclohexene,
vinylcyclohexene diepoxide, bromopropane), alkylating agents (Myleran, trimethylene-
melamin, isopropyl methanesulfonate), methanesulfonate, busulfan, urethane,
procarbazine HCL, dibromochloropropane, bleomycin, and others (Hoyer 2004). The
majority of chemicals used to induce ovarian cancer also cause follicle depletion.
Likewise, chemicals that cause follicle depletion frequently also cause ovarian tumor
development. Studying cancer development in follicle deplete ovaries is important for
translating findings to humans because the majority women with ovarian cancer are
diagnosed after menopause.

1.2 Medical Imaging

Medical imaging includes a variety of technologies used to diagnose, monitor or treat
conditions in the human body. Numerous different imaging methods can be used to
interrogate different physical phenomena. The majority of medical imaging modalities
use waves to collect information from the body. X-ray, computed tomography (CT),
magnetic resonance imaging (MRI), single-photon emission computed tomography
(SPECT), positron emission tomography (PET) and some types of optical imaging all
visualize the transmission of different types of electromagnetic waves through the body.
Ultrasound and optical methods such as OCT visualize waves reflected from the body.
MRI collects information from radiofrequency waves emitted from the body. The
principles behind each technology are different, lending to the ability of each technology
to collect unique information. In the text below, common medical imaging modalities
used in the clinical setting or for research purposes are summarized. Additionally, their
utility for detection of ovarian cancer is described. The advantages, disadvantages and important specifications of each technique are summarized in table 1.2.

1.2.1 X-rays and Computed Tomography

Projection radiography and computed tomography (also called a CT scan or computed axial tomography [CAT] scan) use x-rays to produce images of the body. Images are created by the selective attenuation of X-rays in different tissues throughout the body. The variety of attenuation coefficients from tissues results in different transmissions of X-rays from the tissues and, subsequently, a non-uniform intensity of X-rays on the scintillator. In order to produce an image that can be seen by eye, the scintillator converts X-rays into visible light which is sent to a piece of film or a digital detector to record the image. The intensity profile produces the contrast allowing the visualization of tissues, for example, bones, the heart and lungs in a chest X-ray.

Where projection radiography obtains two dimensional projection images by using a stationary cone beam and single detector, computed tomography obtains two dimensional images by using either a collimated beam or a cone beam and multiple detectors that rotate around a stationary object, allowing for the collection of many projections at different angles. A 3D volume can then be obtained by moving the patient through the scanner. In helical CT the collimated x-ray source and detectors continuously rotate as the patient moves through the CT, producing a helix of images. In multi-slice CT the cone beam x-ray source and rows of detectors rotate as the patient moves through the scanner. After the digital projections are recorded, the images are reconstructed into a 3D volume
that can be viewed as multiple 2D slices. In CT, the spatial resolution is primarily
determined by the size and number of pixels on the detector and the source-to-object
distance (Prince 2006). Clinical CTs generally have resolution around 2 mm (Kalender
2006).

Computed Tomography of Ovarian Tissue

CT is used in ovarian cancer to determine the size of lesions (generally larger than 4 cm),
the type of lesions (solid vs. cystic components) and the stage of disease. Further, CT is
the preferred method to examine the extent of disease and optimal cytoreduction in
metastatic ovarian cancer. Studies have shown that visualization of tumor involvement
with the diaphragm and large bowel on CT predicts suboptimal cytoreduction, resulting
in a decision to do chemotherapy first. CT is recommended for staging but not screening
due to high specificity (92%) and low sensitivity (50%) (Forstner 1995). Many ovarian
tumors are found incidentally on CT and, in that case, ultrasound is generally performed
for follow up.

1.2.2 Nuclear Medical Imaging

In nuclear medical imaging the emission of electromagnetic waves (gamma rays) from
radioactive tracers are detected. The tracers must be injected, inhaled or swallowed. After
entering the body, the compounds will accumulate selectively based on the biological
carrier molecule used in the tracer. The radionuclide (linked to the biological carrier
molecule) undergoes radioactive decay, producing a gamma ray. The gamma rays emitted
are proportional to the amount of tracer in the area. The amount of tracer that
accumulates is often related to the function of the organ and nuclear medical imaging is therefore a functional imaging method (Prince 2006).

In conventional radionuclide imaging, specialized scintillation detectors called Anger cameras are used to detect single gamma rays after they are emitted from the tracer in the body and, as was described in conventional X-ray, 2D projection images are formed (Prince 2006). Also like conventional X-ray, gamma rays are attenuated as they pass through the body. However, rather than being the basis for contrast as in X-ray, the attenuation of the gamma rays confounds the concentration of the tracer by reducing the detected intensity. Two types of radionuclide imaging that allow collection of 3D data sets, single-photon emission computed tomography (SPECT) and positron emission tomography (PET), are described below.

Single-photon Emission Computed Tomography

Similarly to X-ray computed tomography, SPECT imaging is performed by rotating a detector (the Anger camera) around the body while moving the body through the scanner. Collimators are used on the cameras to restrict the angle of the collected photons that reach the detector and, subsequently, data are reconstructed to get a 3D image volume. The photons that are collected are gamma rays that are emitted directly from the radiotracers (typically Tc-99m, I-125, or I-131) that are introduced into the body. Clinical SPECT resolution is generally around 10 mm but is only limited by technology (collimator, detector, etc.); thus, many research SPECT systems have much higher resolution, in the hundreds of microns (Barrett 2008, Knoll 1983).
Positron Emission Tomography

PET differs from SPECT in the type of radiotracers used and the gamma ray detection. The radionuclide tracers used in PET (commonly F-18, Ga-68) undergo decay which produces a positron (rather than a gamma ray photon) (Meuhlelehner 2006). The positron travels a short distance and then annihilates with an electron in the tissue, producing two gamma rays travelling in opposing directions. The two gamma rays from a single molecule are then detected by Anger cameras oriented opposite each other. This method makes the exact location of the molecule (along a line) known and thus makes accurate reconstruction easier. A specialized type of PET, called time-of-flight PET, measures the time difference it takes the anti-parallel gamma rays to reach their respective detectors; this method results in the ability to more precisely locate the annihilation event (Frangioni 2007). The theoretical resolution limitation is the annihilation distance, which is unknown but can be estimated based on tissue density (annihilation distances are longer in less dense tissues) (Frangioni 2007). The best achievable resolution is between 1-3 mm (Muehllehner 2006).

Nuclear Medical Imaging of Ovarian Tissue

SPECT is not used clinically for ovarian cancer detection. PET has been used clinically and is usually paired with CT imaging. One study found that combined PET-CT had a sensitivity of 100% and specificity of 92.5%, an improvement over using CT alone (Risum 2007). However, in that study few early stage cancers were found, partly because the majority of patients presented with late stage disease. Further, other studies have
shown that PET-CT resulted in many false negatives (for early stage and borderline ovarian cancers) as well as many false positives, resulting in sensitivity of 52-58% and specificity of 76-78% (Iyer 2010). PET-CT is more commonly used to detect metastasis or recurrent ovarian cancer, rather than primary ovarian cancer (Gu 2009).

One common tracer used for cancer imaging in PET is radioactively labeled glucose (FDG-PET). Glucose is transported to areas with high glycolytic activity and becomes trapped in areas of highly active cells, like tumors (Yamamoto 2008). FDG-PET has shown some success in detecting tumors in post-menopausal women but is not good for pre-menopausal women because natural (healthy) changes in ovarian tissue such as formation of a corpus luteum cyst results in high glycolytic activity and may be mistaken for a tumor (Shaaban 2009).

1.2.4 Magnetic Resonance Imaging

MRI uses the phenomenon of nuclear magnetic resonance to probe hydrogen atoms in the body. First a strong external magnetic field, ranging from 0.5 to 7 Tesla (thousands of times stronger than the Earth’s magnetic field), is applied to align the spins of the protons in the hydrogen atoms’ nuclei along the magnetic field. Next, radio frequency pulses from a radio frequency coil are used to tip the direction of precession of the proton away from the magnetic field. The magnetization vector of the proton then has a transverse magnetization component and a longitudinal magnetization component. The precession of the proton around the applied magnetic field eventually decays due to transverse (spin-spin) relaxation, caused by perturbations in the magnetic field from nearby spins, and
longitudinal (spin-lattice) relaxation, caused by the exponential recovery of the longitudinal magnetization to the direction of the externally applied field. As the protons re-align to the induced magnetic field their individual transverse magnetizations generate a unique radio-frequency that is measured with a radio frequency coil. The MRI data collected are scans of Fourier space and inverse Fourier transforms are performed to create an image. Owing to the fact that MRI data are collected in Fourier space, the resolution of MRI depends on the highest frequency collected, determined by the pulse sequence. Standard MRI scanners have a resolution of 1-2 mm (Prince 2006, Pykett 1982).

In MRI a variety of RF pulse sequences can be used to change the contrast of tissues. The intrinsic tissue properties that influence nuclear magnetic resonance are proton density, T1 and T2. In proton density-weighted MRI the intensity of the image is proportional to the number of protons (hydrogen nuclei) in the tissue. In T2-weighted images the intensity relates to the transverse relaxation times. In T1-weighted images the intensity is based on the longitudinal magnetization (Prince 2006).

Magnetic Resonance Imaging of Ovarian Tissue

Studies using MRI for ovarian cancer have found that MRI is not particularly good at detecting early stage disease but is accurate for detecting late stage (malignant) disease (Kurtz 1999). This is because with large masses, MRI is especially good at differentiating between benign and malignant tumors owing to the ability to distinguish blood and fat from solid components (Shaaban 2009). One study found that MRI performed similar to
or better than CT or ultrasound in differentiating ovarian cancers. In that study, overall specificities and sensitivities, respectively, were 88% & 98% for MRI, 89% & 92% for CT and 96% & 75% for ultrasound (Kurtz 1999). Another study found MRI sensitivity and specificity as 96.6% and 83.7%, respectively (Sohaib 2005). Thus, MRI is generally used for determination of the type of mass and not for initial screening (Iyer 2010).

1.2.5 Ultrasound

Ultrasound images are produced by using a transducer that puts pulsed high-frequency sound waves (much higher frequencies than can be heard by ear) into the body and subsequently collects the reflected waves, called echoes. By counting the time-of-return of the echoes, the distance into the body from which the wave was reflected can be determined. Additionally, the amplitude of the echo provides information about the tissue properties. A-mode ultrasound uses a single point of sound waves to retrieve a one-dimensional depth scan (not a 2D image). M-mode ultrasound is a repetition of A-mode waves, used to determine time varying structures in a single location. B-mode imaging uses an arc of sound waves to get 2D cross-sectional images, as are seen in sonograms (Prince 2006). Ultrasound can also be used for Doppler imaging, which uses the Doppler effect (frequency shifts caused by reflection of waves off of moving objects) to visualize blood flow (Prince 2006, Wells 2006). Clinical ultrasound systems typically operate in the 10 MHz range, giving resolution around 150 μm (Fujimoto 2000, Rumack 2010). Better resolutions can be achieved by using higher frequency sound waves, but this
decreases the imaging depth. For example, using 100 MHz can achieve 20 µm resolution, but only images a few millimeters deep (Fujimoto 2000).

Ultrasound of Ovarian Tissue

Ultrasound has been the standard imaging technique for detection of ovarian cancer. In general the ovaries are assessed for volume, solid non-fatty components and cystic components and increased blood flow (using Doppler ultrasound) (Shaaban 2009). Additionally, lymph nodes are assessed for size to determine likelihood of malignancy (Shaaban 2009). Most studies using ultrasound (transabdominal and transvaginal) for detection and screening have found that the sensitivity to cancer is high, however, the majority are found at late stages. Additionally, the specificity is low, leading to many false positives, which lead to many unnecessary surgeries (Karlan 1994, van Nagell 2000, Campbell 1988, Sohaib 2005). Most importantly, screening with ultrasound (combined with CA-125 screening) does not reduce ovarian cancer mortality (Buys 2005, Buys 2011).

1.2.6 Optical Imaging

Optical imaging includes a number of different methods which use light and optics to get information about biological systems. The types of light used range from ultraviolet to infrared. The principles used in optical imaging can be categorized broadly into absorption and scattering based methods. In absorption, a molecule intakes the energy from light and either converts it to thermal energy or re-emits the some of the energy (generally at a longer wavelength) in an event called fluorescence. In scattering, index of
refraction changes in tissue cause the direction of light propagation to change. The majority of scattering events are elastic, in which the scattered photon keeps the same frequency. However, there also exists inelastic scattering in which the scattered photon is a different frequency than the incident photon (Povoski 2007, Fowles 1968). Each optical imaging modality creates contrast by recording the effects of absorption or scattering of photons.

1.2.6.1 Optical Coherence Tomography

Optical coherence tomography is an imaging technique that obtains depth-resolved images. Single axial (depth) scans are obtained by detecting reflected light from scatterers (index of refraction mismatches) at different depths in the tissue. Tissue is then scanned laterally to create cross-sectional images. The ability to time the photons and locate the depth that the back-scattered photon came from requires a low-coherence light source and the technique of interferometry. A low-coherence light source is one that has a short distance over which light remains coherent (has the same constant relative phase). OCT uses near infrared light that is typically between 800 nm and 1300 nm central wavelength, with a large bandwidth (generally around 100-200 nm). OCT is frequently compared to ultrasound due to the method of timing reflections. However, light travels too quickly to time the reflected waves, as is done with a transducer in ultrasound, thus interferometry must be used. Interferometry is a technique which allows the backscattering events to be timed in order to locate the specific depth at which they occurred.
In an interferometer, source light is separated into a reference arm and a sample arm using a beam splitter. In the reference arm, light back reflects from a mirror at the end of the arm. In the sample, light back reflects due to changes in optical properties of the microstructural features in the tissue, which can be thought of as a multitude of individual scatterers. Light from the sample and reference arms are then recombined at the beam splitter and imaged on the detector and the interference of the two beams is used to determine the location of reflectors in the tissue. When low-coherence light or short pulses are used, interference of the reference and sample beams occurs when the path lengths are within the coherence length of the light source.

The detected signal is the interfered electric fields from the sample and reference arms. The temporal coherence length is equal to the FWHM (spatial width) of the envelope of the autocorrelation function and determines the axial resolution (Fujimoto 2000, Fujimoto 2001). The temporal coherence length, and therefore axial resolution, is determined by the source central wavelength and bandwidth (equation 1) (Fujimoto 2000). A shorter central wavelength results in improved axial resolution, but is a tradeoff with imaging depth because shorter wavelengths have shallower penetration. A larger bandwidth also results in improved axial resolution. Common OCT system axial resolutions are on the order of 10 microns. However, using KLM lasers with 260 nm bandwidth (centered at 800 nm) and supercontinuum sources with 325 nm bandwidth (centered at 725 nm) and 350 nm bandwidth (centered at 1130 nm), sub-micron axial resolutions have been achieved (Unterhuber 2004). The transverse resolution is determined by the numerical aperture of the imaging optics (equation 2) (Fujimoto 2000).
The transverse resolution can be improved by decreasing the focal length or increasing the entrance pupil diameter of the objective.

\[ z = \left( \frac{2 \ln 2}{\pi} \right) \left( \frac{\lambda^2}{\Delta \lambda} \right) \]  

[Equation 1]

Where \( z \) is the axial resolution (equals the coherence length, also the FWHM of the autocorrelation function), \( \lambda \) is the central wavelength of the source and \( \Delta \lambda \) is the source bandwidth.

\[ x = \left( \frac{4 \lambda}{\pi} \right) \left( \frac{f}{d} \right) \]  

[Equation 2]

Where \( x \) is the transverse resolution, \( \lambda \) is the central wavelength of the source, \( f \) is the focal length of the objective lens and \( d \) is the entrance pupil diameter (determined by the NA of the imaging optics).

The main advantages of OCT are the non-contact, non-destructive nature of imaging, the high resolution and the large imaging depth (when compared to other optical imaging modalities). Deeper imaging is achieved by using NIR light, whereas other optical techniques, like confocal microscopy, commonly use visible or UV light. Imaging depth of OCT is generally 2-3 mm (Fujimoto 2000). This depth penetration plus the ability to scan laterally makes OCT a good candidate for optical biopsy because the volumes imaged are the same size as the amount of tissue typically removed during a traditional biopsy. Additionally, NIR light can be less damaging than other wavelengths of light (such as UV) and NIR light is non-ionizing radiation, making it safer than techniques like CT. OCT also has an advantage over ultrasound in that OCT does not need to contact the tissue, making it useful for imaging tissues where air is present or where non-contact is
preferential, such as in imaging the retina. Two different OCT implementations are described below.

Time Domain OCT

Time domain OCT is performed by scanning the reference arm to build up a depth scan. When a low-coherence light source is used, interference only occurs when the path lengths from the two arms are within the coherence length of the source. Thus, by moving the mirror in the reference arm such that the path length changes, interference occurs from scatterers in the sample arm that are located at the same path length. The light from the reference and sample interfere to create an interference fringe pattern with the peak of the envelope when the path lengths are exactly matched. The interference signal at the detector is given by equation 3 (Leitgeb 2003). By demodulating the interference, the location of the scatterers is determined.

\[
\langle S_{TD}^2 \rangle = 2S^2P_o^2\gamma_r\gamma_sR_rR_s
\]  

[Equation 3]

\(\langle S_{TD}^2 \rangle\) is the interference signal on the detector in a time domain system, \(S\) is the detector responsitivity, \(P_o\) is the optical power, \(\gamma_r\) and \(\gamma_s\) are the fraction of input power leaving the reference and sample arm, respectively, and \(R_r\) and \(R_s\) are the reflectivity of the reference mirror and sample, respectively.

Fourier Domain OCT

Fourier (frequency) domain OCT includes both spatially encoded frequency domain OCT and time encoded frequency domain OCT. In these methods spectral separation is used to collect the interference from the sample and reference arms without the need to scan the
location of the reference arm. In Fourier domain OCT, the optical path distance in the reference arm is set so that it is the same optical path distance to just above the sample in the sample arm. The light in the reference arm reflects off a mirror and the light in the sample arm continues into the sample and is back reflected by scatterers at multiple depths in the sample. The back reflected light contains amplitude and frequency information from each scatterer. The interference of light from the reference and sample arms is then wavelength dependent. The interference signal on the detector is given by equation 4 (Leitgeb 2003). Taking the Fourier transform of the interference gives the equivalent time domain signal and, thus, the location of the scatterers is determined.

Fourier domain OCT has a time advantage over time domain OCT because in Fourier domain OCT the reference arm is not scanned as in time domain OCT. Additionally, frequency domain OCT has a sensitivity (determined by the signal to noise ratio) advantage over time domain OCT (Leitgeb 2003, Choma 2003).

\[
S(v_0)_{FD} = \frac{\rho \eta \tau}{h v_o} 2P(v_o)\sqrt{\gamma_r \gamma_s R_r R_s}
\]  

[Equation 4]

\(S(v_0)_{FD}\) is the interference signal on the detector in a Fourier domain system, \(\rho\) is the spectrometer efficiency, \(\eta\) is the detector quantum efficiency, \(\tau\) is the exposure time, \(h\) is Planck’s constant, \(v_o\) is the center frequency of the light source spectrum, \(P\) is the optical power, \(\gamma_r\) and \(\gamma_s\) are the fraction of input power leaving the reference and sample arm, respectively, and \(R_r\) and \(R_s\) are the reflectivity of the reference mirror and sample, respectively.

Spatially Encoded Frequency Domain OCT

Spatially encoded frequency domain OCT (SEFD-OCT), also called spectral domain OCT, collects information encoded in frequency rather than in time. This is possible
because the interference fringe envelope is the Fourier transform of the power spectrum of the light source. In SEFD-OCT, light is collected as a function of wavelength by using spectrally separated detectors. The interfered light from the reference and sample arms are passed through a grating to separate the wavelengths. In general, the wavelengths are then collected by a linear detector array, resulting in faster imaging because the depth scan is acquired without scanning the reference arm, as in time domain OCT. Taking the Fourier transform of the data gives the equivalent time-domain data.

Time Encoded Frequency Domain OCT

In time encoded frequency domain OCT (TEFD-OCT), also called swept source OCT, a swept wavelength source sweeps through frequencies in the bandwidth of the source such that at any instant in time it appears as a discrete set of wavelengths. The path difference in the arms is thus encoded as a temporal frequency (spectral components are encoded in time instead of in space as in SEFD-OCT). The interference signal is generated in discrete wavelength steps and the signal is reconstructed before taking the Fourier transform to get the equivalent time domain data.

OCT of Ovarian Tissue

OCT has been used to image microscopic structures in mouse, rat and human ovaries both \textit{ex vivo} and \textit{in vivo}. Further, many studies have shown that OCT can detect differences in normal and abnormal or malignant ovarian tissue. OCT successfully visualized a primordial ovarian follicle and cysts in \textit{ex vivo} human ovary (Boppart 1997, Boppart 1999). In another study, OCT showed that patterns of back scattered intensity
were noticeably different between normal and abnormal *ex vivo* ovarian tissue samples (Brewer 2004). OCT has also been used to quantitatively estimate the optical scattering coefficient of fresh, *ex vivo* normal and malignant ovarian tissue. Examination of scattering coefficients found that malignant tissue had a significantly lower scattering coefficient than normal tissue, resulting from the decrease in collagen area fraction of malignant tissue (Yang 2011a). OCT imaging in rat ovaries was able to identify follicles in various stages, corpora lutea, epithelial invaginations, ovarian bursa, cysts and tumors (Hariri 2010, Kanter 2006). Additional ovarian tissue studies have combined OCT with other methods such as scintillators, photoacoustic imaging and ultrasound, to try to improve cancer detection (Gamelin 2009, Yang 2011b).

In a clinical study, Hariri et al. used a laparoscopic OCT to image human ovaries *in vivo*. They found that OCT was safe for *in vivo* laparoscopic imaging and OCT was able to visualize microstructural features of the ovary such as ovarian stroma, corpus albicans, epithelial invaginations and cysts in normal ovary, an increase in blood vessels and cysts in ovaries with endometriosis and cysts and areas of malignant cells in ovarian carcinoma (Hariri 2009).

1.2.6.2 Photoacoustic Imaging

Photoacoustic imaging (PAI) is a technique that combines the principles of optical imaging and ultrasound. In PAI, a laser is shown into tissue causing a slight temperature increase that results in thermoelastic expansion of the tissue. The thermoelastic expansion causes formation of acoustic waves which are collected by ultrasound transducers and
reconstruction is performed to visualize the light distribution levels in the tissue. This technique primarily relies on absorption of light by blood vessels and is useful for finding tumors due to the increase in angiogenesis in tumors.

Photoacoustic Imaging of Ovarian Tissue

A couple studies have been done on ex vivo human and animal ovarian samples using PAI (Aguirre 2011, Yang 2011a, Yang 2011b). One study on ex vivo human ovarian tissue found that there was a lot of variation in absorption of ovaries, but malignant ovaries generally had higher absorption than normal (Aguirre 2011).

1.2.6.3 Fluorescence Imaging

Fluorescence imaging uses endogenous or exogenous fluorophores to obtain tissue contrast. Each fluorophore has a specific excitation and emission spectra that can be used to identify the molecule. Fluorescence imaging is typically used to identify morphologic or fluorescence spectra changes in tissues resulting from disease.

Fluorescence Imaging of Ovarian Tissue

Using fluorescence spectroscopy, Brewer et al. found notable changes in the endogenous fluorescence spectra of normal and cancerous ovary. These changes included increased 380nm fluorescence emission at 330 nm excitation, increased 460 nm emission at 330-360 nm excitation, and decreased 460 nm emission at 400-430 nm excitation in carcinoma, as compared to normal ovary (Brewer 2001). Fluorescence imaging of targeted fluorophores such as folate conjugated to FITC for targeting folate receptor, has
been used in cytoreductive surgery of ovarian cancer (van Dam 2011). Also, fluorescent photodynamic therapy agents 5-aminolevulinic acid and benzoporphyin-derivative monoacid ring A have been used in metastatic ovarian cancer detection and treatment (Loning 2004, Zhong 2009).

1.2.6.4 Confocal Microscopy

Laser scanning confocal microscopy (confocal microscopy) is a microscopy method that allows collection of deep serial sections in tissue by reducing the collection of background information outside of the focal plane. The procedure includes illuminating a point on the sample by passing laser light through an objective lens, subsequently separating the reflected (or fluorescent) light (coming from the sample) from the illumination light using a dichromatic mirror and filter and then passing the light through a pinhole in a conjugate image plane of the system. The pinhole lets through the light that was in focus on the sample and blocks all the out of focus light that falls outside of the pinhole. The light that passes through the pinhole is then collected by a detector. In order to build up a 2D image, the laser is raster scanned across the sample. Then to get serial sections, the focal point is moved up or down in the tissue (by moving the objective or the tissue) and another scan is completed. Confocal microscopy is generally used in fluorescence mode with exogenous agents because endogenous fluorescence is too weak to see. Common exogenous contrast agents include fluorescein, DAPI, Texas red, acridine orange, TRITC, and GFP. The resolution of confocal microscopy is limited by the NA of the imaging optics and the wavelength of the light source. Using high NA
optics, sub-micron resolution has been achieved. The depth penetration is limited by the wavelength of the source and maximum imaging depths are around 100 µm (Shotton 1989).

Confocal Microscopy of Ovarian Tissue

Confocal microscopy has been used to examine animal and human ovaries. Fluorescence confocal microscopy was able to successfully visualize cell morphology in the epithelial and stromal layers of *ex vivo* human samples (using acridine orange fluorescent dye) (Brewer 2004). Additionally, confocal microlaparoscopes have been developed for *in vivo* fluorescence imaging of human ovary. Both fluorescein, an approved dye for human use, and acridine orange, not an approved dye, have been used for *in vivo* fluorescence confocal microscopy of ovaries that were removed after *in vivo* imaging. *In vivo* and *ex vivo* imaging with these dyes allowed visualization of the epithelial and stromal cells and identified changes in the epithelium among normal, adenocarcinoma and endometriosis specimens (Tanbakuchi 2008, Tanbakuchi 2009).

1.2.6.5 Multiphoton Microscopy

Multiphoton microscopy (MPM) includes a number of non-linear imaging techniques in which multiple photons’ energies are combined in absorption-emission or scattering events. At low power these non-linear events are rare. However, by creating a small focal volume with high instantaneous power, the probability of non-linear events can be greatly increased such that the non-linear events happen at detectable levels. The probability of occurrence of non-linear phenomena depends on the square of the incident photon flux
and therefore, decreases rapidly outside of the focal volume, lending to the great depth sectioning and contrast without the need for a pinhole. The small focal volume of instantaneous high energy is achieved by using a high NA objective and a femtosecond pulsed laser.

The high NA objective creates a very small focal volume where the non-linear events occur. Thus, a pinhole is not required. The high NA objective provides excellent (sub-micron) lateral and axial resolution and greatly reduces out of focus photo-bleaching. The femtosecond pulsed laser creates the high instantaneous energy, while still keeping average energy low, so as to not damage the tissue being imaged. Additionally, the laser light used in MPM is generally in the near infrared, typically causing less damage than UV wavelengths that are frequently used in confocal microscopy. Furthermore, NIR light allows for deeper penetration into tissues, as compared to methods that use incident light in the visible and UV wavelengths, due to reduced scattering and absorption. The most common laser used in MPM is titanium-sapphire with 100-fs pulses at a repetition rate of around 80 MHz. Further, these lasers can be tuned from 700-1000 nm, corresponding to the optimal optical penetration for tissue. Yet another advantage of using NIR light is the spectral separation of the incident and emitted light that allows separation of the signal from the laser light with minimal losses.

Similarly to confocal microscopy, to build a 2D image, the focal point is raster scanned across the tissue and to build a 3D volume the focal point is moved up or down in the tissue by moving the tissue or the objective. Depth of imaging is generally in the
hundreds of microns depending on wavelength and tissue type. Depths of 500 µm have been achieved in transparent tissues like the brain, but in ovarian tissue the maximum depth is generally around 200 µm (So 2000, Zipfel 2003, Williams 1994).

Many phenomena can be collected in multiphoton imaging including second harmonic generation (SHG), third harmonic generation, two-photon excited fluorescence (TPEF), three photon excited fluorescence. Further, multiple phenomena can be collected at once by spectrally separating the emission (using dichroic and band pass filters that are optimized for the spectrum of each phenomena) and subsequently collecting the separated spectra on multiple detectors. The two most commonly collected MPM phenomena, second harmonic generation (SHG) and two-photon excited fluorescence (TPEF), are described below.

Two-photon Excited Fluorescence

In two-photon excitation a molecule near-simultaneously absorbs two photons. The two photon absorption induces a transition to an excited electronic state. When the molecule relaxes to its electronic ground state a single photon is emitted (fluorescence). Since the energies of two photons were combined, the emitted photon is usually at a higher energy (shorter wavelength) than the absorbed light. The number of photons absorbed per pulse by each fluorophore is described by equation 5 (So 2000). Increasing the power, decreasing the pulse duration and increasing the NA (decreasing the focal volume) are methods of increasing photon absorption and increase the number of non-linear events, thereby increasing the detected signal.
\[ n \approx \frac{p^2 \delta (NA)^2}{\tau f^2 2hc\lambda} \]  

[Equation 5]

Where \( n \) is the number of photons absorbed, \( p \) is the average laser intensity, \( \delta \) is the fluorophore’s two-photon absorption at wavelength \( \lambda \), \( \tau \) is the pulse duration, \( f \) is the laser repetition rate, \( NA \) is the numerical aperture of the focusing objective, \( h \) is Planck’s constant and \( c \) is the speed of light.

TPEF allows excitation of multiple different fluorophores in the focal volume at once and the emissions of spectrally separated fluorophores can be collected separately using dichroic and band pass filters. Common endogenous fluorophores from which TPEF is collected include nicotinamides (NADH), flavins (FAD), structural proteins (collagen, elastin) and other proteins containing amino acids tryptophan & tyrosine. It should be noted, the quantum mechanics of one and two photon excitation are not the same. Thus, using exactly twice the wavelength of the one photon absorption maximum for two photon excitation may not be optimal.

Second Harmonic Generation

SHG imaging is a non-linear imaging technique in which two photons are combined into one photon during scattering in a non-centrosymmetric material. Due to this scattering event the emitted photon is at exactly half the wavelength (twice the energy) of the incident photons.

SHG occurs when large electric fields are present in a molecule with nonlinear polarizability. The electric field induces a polarization in the sample that is described by equation 6 (Yazdanfar 2004, So 2000).
\[ P = \epsilon (X^{(1)}E + X^{(2)}EE^* + \ldots) \]  \[\text{[Equation 6]}\]

Where \( P \) is the polarization, \( \epsilon \) is the dielectric constant, \( X^{(n)} \) is the \( n \)th-order susceptibility tensor and \( E \) is the electric field vector.

The second order susceptibility tensor \( X^{(2)} \) (in equation 6) depends on the density of molecules and the orientational average of the molecular hyperpolarizability (Campagnola 2011). Note the orientational average—this means that hyperpolarizability of molecules with inversion symmetry cancel. Thus, only molecules with non-centrosymmetric structure have a non-zero second-order susceptibility tensor and will produce a coherent electric field output at exactly twice the frequency of the incident wave. The intensity of the second harmonic signal increases non-linearly with increased incident energy (SHG signal is proportional to the square of the laser pulse energy), achieved with high power short pulses (Campagnola 2003). Additionally, the hyperpolarizability is extremely strong when the frequency of the incident light is in resonance with an electronic absorption band and results in a large intensity SHG signal. Similarly to TPEF, SHG has a non-linear (quadratic) dependency on the electric field amplitude and signal falls off quickly outside of the focal volume, providing excellent depth sectioning capability (Campagnola 2011).

Biological molecules that are non-centrosymmetric and produce SHG include collagen, myosin and microtubules. Collagen is the most abundant protein in the body and frequently undergoes remodeling in and near tumors and is therefore frequently studied for cancer detection methods. Collagen molecules are made up of triple helix chains of repeating sequences of amino acids. The collagen molecules aggregate together, forming
collagen fibrils, which are bundled together to form collagen fibers. The SHG signal comes from coherent amplification by peptide bonds along the length of the fibrils (Campagnola 2011). SHG from collagen results in forward and backward scattering, with the ratio being influenced by fiber size and packing (LaComb 2008).

MPM of Ovarian Tissue

SHG and TPEF imaging has been shown useful for discriminating normal from cancerous ovarian tissue in human tissue *ex vivo* and mouse tissue both *ex vivo* and *in vivo*. Images of *ex vivo* human ovary biopsies showed that the collagen structure appeared more wavy in malignant than in normal ovaries and TPEF imaging showed a change in cellular morphology from uniform epithelial layer in normal to non-uniform larger cells in abnormal tissue (Kirkpatrick 2007, Williams 2010). Similar structural changes including variable cell size, multiple epithelial layers and change in collagen structure, were observed from TPEF and SHG imaging in fixed, stained, human ovarian tissue samples (Adur 2011). Nadiarnykh et al. found that malignant ovaries had denser collagen structure and higher regularity in fibril and fiber structure and higher scattering coefficient, resulting in less forward scattering than normal tissue (Nadiarnykh 2010).

Examination of endogenous fluorescence spectra has shown a red shift in fluorescence of ovarian cancer specimens (Williams 2010). In another study, collection of multiwavelength TPEF (NADH/FAD) allowed estimation of cellular redox ratios, which were more variable for cancer than normal ovary samples (Kirkpatrick 2007).
Williams et al. developed a laparoscopic probe for *in vivo* MPM (Williams 2010). Using the laparoscopic probe, they performed *ex vivo* and *in vivo* imaging in a mouse model of ovarian cancer (TgMISIIR-Tag) and saw from TPEF that ovarian carcinomas had more densely packed and atypical epithelial cells. With SHG, they found the opposite as others in that collagen appeared wavy in normal and straight in cancer. Other small rigid multiphoton endoscopes have been developed for *in vivo* imaging, and have thus far primarily been used for skin imaging in both animals and humans (Kim 2008, Konig 2001, Konig 2007, Balu 2013).

1.2.7 Contrast Agents for Medical Imaging

With the majority of the imaging modalities described here the main contrast is endogenous, meaning contrast is derived from native tissue components (e.g. hydrogen protons in MRI, NADH in fluorescence). Using endogenous contrast is usually preferred because there is less risk and generally less time involved than when administering exogenous contrast agents. However, all of these techniques can achieve improved contrast by using exogenous contrast agents specific to the imaging technique. Additionally, many contrast agents can be used not only for disease detection, but also for therapy. For example, the photosensitive agent benzoporphyrin-derivative monoacid ring A (BPD-MA) is used for improved detection with fluorescence imaging and photodynamic therapy in ovarian cancer (Zhong 2009).

The majority of contrast agents are non-specific to disease and provide contrast by conforming to the areas in which they are introduced or by accumulating in diseased
areas due to changes in flow, such as angiogenesis in tumors. Examples of non-specific contrast agents include gadolinium in MRI, iodine in x-ray angiography, barium in gastrointestinal CT, and fluorescein and indocyanine green in optical imaging. However, more advanced contrast agents, which target specific cell receptors in cancer, are currently being developed and are likely to greatly improve cancer detection and treatment. The targeting approach includes selection of a contrast agent appropriate for the imaging technique and then attaching to the contrast agent a ligand that will attach only to the receptors of interest. This technique provides improved contrast at only the receptors of interest and allows for targeted therapy. Further, by using multiple, unique ligands, multiple receptors can be targeted at once to provide a combination of different types of contrast and targeted therapies. Popular ligands of study in ovarian cancer include those that bind to receptors that are overexpressed in ovarian cancer, such as folate receptor and epidermal growth factor receptor (EGFR) (Konda 2001, Moscatello 1995, Kalli 2008, van Dam 2011). One recent study used folate conjugated to fluorescein isothiocyanate (FITC) for folate receptor alpha targeted contrast (van Dam 2011). This study found that real-time multispectral fluorescence imaging of folate receptor alpha improved intraoperative staging and allowed for more radical cytoreductive surgery (van Dam 2011). With the recent developments in genetic mutations leading to ovarian cancer, additional receptors of interest in ovarian cancer are likely to be found in the future.
<table>
<thead>
<tr>
<th>Modality</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Resolution</th>
<th>Imaging depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>Whole body imaging</td>
<td>Ionizing radiation</td>
<td>2 mm</td>
<td>&gt;10 cm</td>
</tr>
<tr>
<td>SPECT</td>
<td>Whole body imaging, Functional imaging</td>
<td>must use exogenous contrast agents, ionizing radiation</td>
<td>10 mm (technology limited)</td>
<td>&gt;10 cm</td>
</tr>
<tr>
<td>PET</td>
<td>Whole body imaging, Functional imaging</td>
<td>must use exogenous contrast agents, ionizing radiation</td>
<td>2-3 mm</td>
<td>&gt;10 cm</td>
</tr>
<tr>
<td>MRI</td>
<td>Many different sequences to adjust contrast for tissue type</td>
<td>Loud, dangerous near ferrous metals</td>
<td>1-2 mm</td>
<td>&gt;10 cm</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>Anatomical and functional information</td>
<td>Difficult to use, must be in contact with tissue</td>
<td>10 µm to 1 mm</td>
<td>2 mm to 10 cm</td>
</tr>
<tr>
<td>OCT</td>
<td>Non-contact, depth sectioning, non-destructive endoscope/laparoscope enabled, compact, portable</td>
<td>Shallow penetration depth</td>
<td>10 µm</td>
<td>2-3 mm</td>
</tr>
<tr>
<td>Confocal</td>
<td>Depth sectioning, non-destructive endoscope/laparoscope enabled, compact, portable</td>
<td>Shallow penetration depth</td>
<td>Sub-micron</td>
<td>100 µm</td>
</tr>
<tr>
<td>MPM</td>
<td>Depth sectioning, non-destructive endoscope/laparoscope enabled, collect multiple phenomena simultaneously</td>
<td>Shallow penetration depth</td>
<td>Sub-micron</td>
<td>500 µm</td>
</tr>
</tbody>
</table>
1.3 Explanation of Dissertation Format

Following the introduction, this dissertation contains an explanation of the present study. The present study section references the appendices that follow. The appendices include three papers (two published and one recently submitted) as well as supporting information for the study such as animal subjects approval, standard operating procedures and lists of important file locations.

For both the first and second papers (appendices A and B, respectively), the author performed all imaging, analysis and prepared the manuscripts.

For the third paper (appendix C), the author completed ovary isolation during surgery, the majority of the imaging and performed the analysis and manuscript preparation.
PRESENT STUDY

2.1 Motivation

Ovarian cancer is deadly due to the poor understanding of disease etiology and progression. The frequent late stage diagnosis makes identification of early markers extremely difficult. The use of an animal model in a controlled environment is one of the best ways to monitor progression of disease that is difficult to observe in humans. High-resolution imaging methods are necessary to see the early cellular-level changes that occur with disease. For this reason, optical imaging is the best medical imaging method. In the present study two optical imaging methods, optical coherence tomography and multiphoton microscopy, are used to examine the microscopic changes that occur in mouse ovarian tumor development.

The main contribution of this work to the field is the identification and quantification of microscopic indicators of disease development that have potential application in detection methods for women at high risk for developing ovarian cancer.

In the following sections three specific aims are discussed. For each aim, the motivation for investigating the aim is explained, the methods and results are outlined and the most important findings from each aim are summarized. Detailed methods, results, and conclusions of the studies for each of the three aims discussed below are presented in the three respective papers appended to this dissertation (Appendices A, B, C). The commercialization potential of OCT and MPM for ovarian cancer detection are described in Appendix D.
2.2 Aim 1: Use second harmonic imaging to quantitatively examine the morphological differences in collagen structure in normal mouse ovarian tissue and mouse ovarian tumors.

Motivation

Past literature has suggested that collagen structure changes occur during disease development. These changes have been observed in human ovaries as wavier collagen fibers in cancer specimens than in normal specimens. However the changes have never been shown in a mouse model of ovarian cancer, nor have they been quantified. Confirming the collagen changes in a mouse model validates the use of the mouse model for studying changes in humans. Quantifying the structural changes in collagen allows study of the magnitude of the difference between normal and cancer and the potential usefulness of collagen structure as a diagnostic indicator.

Summary

For this imaging study, mice were dosed with VCD and/or DMBA to cause follicular atresia and tumor development. Second harmonic generation imaging of collagen was performed on control and dosed mouse ovarian tissue \textit{ex vivo}. Quantitative analysis of SHG image data was performed by calculating frequency band ratios of the 2D discrete Fourier Transform and texture parameters from gray-level co-occurrence matrices. Images were grouped according to histologic diagnosis as normal, non-carcinoma tumors and adenocarcinoma. A support vector machine was used to find the image data
parameters that best separated adenocarcinoma from normal and from non-carcinoma (including normal and non-cancer tumors). Refer to Appendix A for the published paper.

Important findings

Visualization of collagen structure by eye in SHG images showed a noticeable difference in collagen structure among normal, non-carcinoma tumors and adenocarcinoma. As was previously seen in human ovaries, normal ovaries had thin, linear collagen fibers and adenocarcinoma had thicker, wavy collagen fibers.

Quantitative analysis showed a decrease in high frequency content of SHG images from normal to adenocarcinoma, and non-cancer to adenocarcinoma. Analysis also showed less variation in gray levels (calculated by GLCM energy) across the image for adenocarcinoma specimens, as compared to normal and non-cancer specimens. Classification of non-carcinoma and adenocarcinoma images based on these two parameters resulted in sensitivity and specificity of 80%. Findings from this study show that quantitative measures of collagen morphology are useful in distinguishing ovarian carcinoma from normal ovary and non-carcinoma tumors.

2.3 Aim 2: Examine the differences in endogenous two-photon fluorescence in normal mouse ovarian tissue and mouse ovarian tumors.

Motivation

Previous fluorescence imaging in the ovary has indicated that fluorescence spectra changes are observed from normal to cancerous ovaries. However, the morphological
changes in fluorescence resulting from ovarian adenocarcinoma have not been examined in detail. Understanding the morphological changes would help develop diagnostic indicators for ovarian cancer.

Summary

Ovaries from control, VCD and/or DMBA dosed mice were imaged with multiphoton microscopy ex vivo. Two-photon excited fluorescence image data were analyzed for changes in fluorescence morphology with disease. Additional fluorescence imaging and specialized stains were performed to identify the type and location of endogenous fluorophores. Punctate and cellular-sized regions of extremely bright fluorescence were segmented from the image. The number and size of punctate and cellular-sized bright regions were calculated. The size and number of regions for normal ovaries at different ages were investigated. Additionally the size and number of regions for normal and various tumor types, including adenocarcinoma, were compared and analyzed for significance. The published paper and standard operating procedures used for this study can be found in Appendix B.

Important findings

TPEF images showed a visible change in cellular morphology with tumor development and an increase in bright fluorescent regions in tumors. Additional fluorescence imaging and specialized staining showed that the bright fluorescence was primarily from lipoproteins located in the interstitial cells (including, but not limited to macrophages) of the ovary.
Analysis of bright regions in normal tissues by age revealed an increase in the number and size of bright regions with aging. This trend follows the known increase in lipoprotein accumulation with age seen in other organs such as the heart. Region analysis of all tissues at all ages showed a trend toward more bright cellular-sized regions and an increase in the size of bright regions in ovarian tumors as compared to normal ovaries. However, the increase was only significant for one tumor type, tubular adenoma. The findings from this study indicate that lipoprotein accumulation is accelerated by disease and increases in lipoprotein may be a useful indicator of ovarian tumor development. However, it must also be noted that increases may be confounded by age.

2.4 Aim 3: Identify changes in ovarian microstructure resulting from disease development by imaging animals in vivo at three time points during a long-term survival study.

Motivation

Very little in vivo image data is available for ovarian cancer. Additionally, time serial longitudinal studies in animals or humans have not been completed. The ability to image in vivo allows for more accurate data regarding the actual tumor environment, without being influenced by excision of the ovary and the resulting hypoxic stress on the tissue. Time serial in vivo imaging also allows visualization of the progression of the disease within the ovary, allowing more accurate visualization of changes within an ovary, as opposed to comparing normal and diseased tissue from separate animals, as is usually done. Time serial imaging within an animal also enables visualization of early changes
with disease development. The ability to image early changes in animals *in vivo* will help toward developing *in vivo* diagnostic tests for women, which would be used to monitor the ovary over time for early disease detection.

Summary

Mouse ovaries were imaged *in vivo* at 6, 11 and 13 months of age. Within that time period, mice were dosed with VCD and/or DMBA (or not dosed as control) to induce tumor development. At each imaging time point the right ovary was accessed through a sterile surgical procedure, secured at the surface of the animal for imaging with MPM and OCT and subsequently returned to anatomical position. Then the incision was closed and allowed to heal before the next imaging time point. After the last imaging time point the ovaries were explanted, imaged *ex vivo*, processed for histology and diagnosed. Time serial OCT and MPM images were inspected for microscopic changes over time in order to identify early microscopic evidence of tumor development. The published paper and standard operating procedures can be found in Appendix C.

Important findings

This study proved the feasibility of imaging mouse ovaries *in vivo* at multiple time points throughout an animal’s lifetime. Evaluation of time serial images confirmed a change in microscopic structure within ovaries over time, during tumor development. Further, early changes were visible in OCT and MPM before changes were visible by eye. Early changes seen by OCT included microscopic changes in surface roughness and scattering homogeneity. Early changes seen with MPM included changes in cellular morphology.
and increases in lipoprotein content of the ovarian tissue, seen with TPEF, and changes in collagen morphology, seen with SHG. The findings from this study indicate that imaging with OCT and MPM enables visualization of early changes during tumor development and a combined OCT-MPM imaging system may be useful in early detection of ovarian cancer in women.

2.5 Conclusion

The work in the present study identified qualitative and quantitative changes during early tumor development. The changes included alterations in collagen structure, increases in endogenous fluorescence from lipoproteins, changes in surface regularity and changes in homogeneity of scattering. Some trends seen in mice in this study, such as changes in collagen morphology with disease and increases in lipoproteins with age, have been seen previously in humans. This validates the use of this mouse model for studying ovarian cancer and indicates that the findings may be useful for early disease detection in humans.
REFERENCES


25. Craig, Z; Davis, J; Marion, S; et al. 7,12-dimethylbenz[a]anthracene induces sertoli-leydig cell tumors in the follicle-depleted ovaries of mice treated with 4-vinylcyclohexene diepoxide. Comparative Medicine 2010; 60(1):10-17.


34. Fredricksen, T. Ovarian tumors of the hen. Environmental Health Perspectives 1987; 73:35-51.


37. Gamelin, J; Yang, Y; Biswal, N; et al. A prototype hybrid intraoperative probe for ovarian cancer detection. Optics Express 2009; 17(9): 7245-7258.


42. Hennessy, B; Coleman, R; Markman, M. Ovarian Cancer. The Lancet 2009; 374(9698):1371-1382.


44. Hoyer, P; Davis, J; Bedrnicek, J; et al. Ovarian neoplasm development by 7,12-dimethylbenz[a]anthracne (DMBA) in a chemically-induced rat model of ovarian failure. Gynecologic Oncology 2009; 112(3): 610-615.


60. LaComb, R; Nadiarnykh, O; Townsend, S; Campagnola, P. Phase matching considerations in second harmonic generation from tissues: effects on emission directionality, conversion efficiency and observed morphology. Optics Communication 2008; 287(7): 1823-1832.


72. Nadiarnykh, O; LaComb, R; Brewer, M; Campagnola, P. Alterations of the extracellular matrix in ovarian cancer studied by second harmonic generation imaging microscopy. BMC Cancer 2010; 10:94.


90. Tanbakuchi, A; Rouse, A; Hatch, K; et al. Clinical evaluation of a confocal microendoscope system for imaging the ovary. SPIE 2008; 6851-03.

91. Tanbakuchi, A; Rouse, A; Hatch, K; et al. Clinical results with acridine orange using a novel laparoscope. SPIE 2009; 7172.


95. Vanderhyden, B; Shaw, T; Ethier, J. Animals models of ovarian cancer. Reproductive Biology and Endocrinology 2003; 1(67).


97. van Nagell Jr., J; DePriest, P; Reedy, M; et al. The efficacy of transvaginal sonographic screening in asymptomatic women at risk for ovarian cancer. Gynecologic Oncology 2000; 77:350-356.


100. Williams, R; Piston, D; Webb, W. Two-photon molecular excitation provides intrinsic 3-dimensional resolution for laser based microscopy and microphotochemistry. FASEB 1994; 8:804-813.


102. Yang, Y; Wang, T; Biswal, N; et al. Optical scattering coefficient estimated by optical coherence tomography correlates with collagen content in ovarian tissue. Journal of Biomedical Optics 2011; 16(9): 090504.
103. Yang, Y; Li, X; Wang, T; et al. Integrated optical coherence tomography, ultrasound and photoacoustic imaging for ovarian tissue characterization. Biomedical Optics Express 2011; 2(9): 2551-2561.


APPENDIX A: ANALYSIS OF SECOND HARMONIC GENERATION MICROSCOPY IN A MOUSE MODEL OF OVARIAN CARCINOMA

A.1 Published Manuscript: Analysis of Second Harmonic Generation Microscopy in a Mouse Model of Ovarian Carcinoma.

The following manuscript has been published in the 17(7) 2012 issue of the Journal of Biomedical Optics.
Title: Analysis of Second Harmonic Generation Microscopy in a Mouse Model of Ovarian Carcinoma

Authors:
Jennifer M. Watson, Photini F. Rice, Samuel L. Marion, Molly A. Brewer, John R. Davis, Jeffrey J. Rodriguez, Urs Utzinger, Patricia B. Hoyer, Jennifer K. Barton

Abstract:

Second harmonic generation (SHG) imaging of mouse ovaries ex vivo was used to detect changes in collagen structure accompanying ovarian cancer development. Dosing with 4-vinylcyclohexene diepoxide and 7, 12—dimethylbenz[a]anthracene resulted in histologically confirmed cases of normal, benign abnormality, dysplasia, and carcinoma. Parameters for each SHG image were calculated using the Fourier transform and gray-level co-occurrence matrices (GLCM). Cancer versus normal and cancer versus all other diagnoses showed the greatest separation using the parameters derived from power in the highest frequency region and GLCM energy. Mixed effects models show that these parameters were significantly different between cancer and normal (p < 0.008). Images were classified with a support vector machine, using 25% of the data for training and 75% for testing. Utilizing all images with signal greater than the noise level, cancer versus not-cancer specimens were classified with 81.2% sensitivity and 80.0% specificity and cancer versus normal specimens were classified with 77.8% sensitivity and 79.3% specificity. Utilizing only images with greater than of 75% of the field of view containing signal improved sensitivity and specificity for cancer versus normal to 81.5% and 81.1%. 
These results suggest that using SHG to visualize collagen structure in ovaries could help with early cancer detection.

Keywords: collagen, ovarian cancer, multiphoton, two-photon, imaging, 7, 12—dimethylbenz[a]anthracene

Introduction

In the U.S. alone there are more than 20,000 new cases of ovarian cancer each year and more than 15,000 deaths per year. The most common type of ovarian malignancy is epithelial cell derived and is more likely to occur in post-menopausal women. Mortality rates are high because an effective screening test does not currently exist. Only 15% of ovarian cancers are found before metastasis has occurred. If ovarian cancer is found and treated before metastasis, the 5-year survival rate is 94% (versus 28% for metastatic disease). Thus, an early diagnostic test to detect pre-malignant changes would save many lives.

Current methods of screening consist of measuring serum levels of cancer antigen 125 (CA-125) and transvaginal ultrasound. Serum levels of CA-125 are often elevated in women with ovarian cancer. However, CA-125 levels are influenced by conditions other than ovarian cancer such as other cancers, lung disease, liver cirrhosis, hysterectomy, obesity and smoking habits. Furthermore, CA-125 is usually only elevated in patients with stage II-IV cancer, not in patients with borderline tumors or stage I ovarian cancer.
Transvaginal ultrasound can be used to visualize both ovaries and evaluate the size of lesions to determine the extent of tumor growth and metastasis.\textsuperscript{14, 15} However, tumor morphology and vascular perfusion, as seen by ultrasound, are not enough to identify abnormalities in ovaries of normal volume or distinguish between benign and malignant tumors.\textsuperscript{16, 17} Furthermore, CA-125 combined with ultrasound does not decrease the number of mortalities resulting from ovarian cancer.\textsuperscript{18, 19}

Due to the low performance of CA-125 and ultrasound in detection of early cancers, women who are at high risk may be advised to undergo a prophylactic salpingo-oophorectomy (removal of the ovaries and fallopian tubes). While this procedure is highly effective at reducing cancer risk, removal of the ovaries is known to increase morbidity and mortality.\textsuperscript{21-24}

Optical methods have been investigated for detection of ovarian cancer. These optical methods include spectroscopy, optical coherence tomography (OCT), confocal microscopy, photoacoustic imaging (PAI) and multiphoton microscopy (MPM). Several studies have been performed utilizing these modalities. However, most comparisons are between normal and advanced cancer because women rarely present with early stage ovarian cancer.

Reflectance and fluorescence spectroscopy can differentiate normal and neoplastic ovarian tissue with good sensitivity and specificity.\textsuperscript{25-29} Limitations of spectroscopy include shallow depth of penetration and low spatial resolution. OCT images of the ovary show details of tissue microstructure such as surface epithelium, follicles, cysts, collagen
bundles and vessels. Furthermore, differences between normal and abnormal/neoplastic ovarian tissue are seen, such as epithelial inclusions, invaginations and differences in attenuation. However, OCT does not have the resolution necessary to visualize dysplasia. Confocal microscopy produces subcellular-resolution images that can be used to identify cancer occurring on the surface of the ovary, but the depth of imaging is limited. Photoacoustic imaging (PAI) has the largest depth of imaging (2-3 cm) and can visualize large structures such as corpora lutea, follicles and blood vessels due to differences in absorption properties. Likewise, malignant and normal ovaries in post-menopausal women can be distinguished by their different absorption properties. However, PAI has relatively low resolution and may be confounded by benign conditions with high vascularity or hemorrhage, or early stage cancers without significant vascularity changes.

Multiphoton microscopy (MPM) can achieve submicron resolution, comparable to a confocal microscope. Due to the decrease in scattering at longer wavelengths, MPM using near infrared light has the ability to image hundreds of microns deeper than confocal microscopy using ultraviolet or visible light. Also, less out-of-focus fluorescence emission is generated, due to the non-linear properties of the multiphoton process, potentially increasing depth of imaging and improving resolution. The resolution limit of MPM depends on the illumination point spread function. The depth limit of MPM depends on the pulse energy, tissue attenuation (from absorption and scattering), and the ratio of collected signal to background signal. In MPM femtosecond pulsed laser light is focused onto the tissue with high numerical aperture (NA) optics, resulting
in high instantaneous power density in a small volume of tissue. In two-photon excited fluorescence (TPEF) two photons are simultaneously absorbed by a fluorophore and then emitted as one photon at a higher frequency than the incident light. In second harmonic generation (SHG), phase matching of photons in non-centrosymmetric structures results in a scattering event in which two photons are combined into one photon at twice the frequency of the incident light. Remitted light from TPEF and SHG are separated using band pass filters and collected with photomultiplier tubes. The laser beam is scanned throughout the tissue volume to create a 3D image set. Because the probability of the multiphoton process is very low outside the small focal volume, very fine sectioning capacity is possible. MPM can be used to see changes in endogenous cellular fluorescence and collagen structure as a result of ovarian cancer.\cite{39-41} SHG shows that normal ovaries have thin collagen fibers organized in a net-like structure whereas malignant ovaries have a denser, wavy collagen structure, possibly resulting from recruitment of activated fibroblasts to the outer rim of the tumor.\cite{39, 41-43} Furthermore, the collagen structure of normal low-risk and normal high-risk post-menopausal ovaries are slightly different.\cite{39} SHG may offer a useful balance of sensitivity, resolution, and depth of imaging.

Imaging ovarian tissue \textit{in vivo} or surgical samples \textit{ex vivo} can provide useful indication of the difference between normal and cancerous ovaries, but it is difficult to ascertain what changes preceding cancer development can be visualized. Women usually present with advanced disease, and the etiology of ovarian cancer is poorly understood. Mouse models of ovarian cancer may provide insight into ovarian cancer development. We
utilize a cancer model in mice that have undergone early ovarian failure because most ovarian cancers arise in post-menopausal women.\textsuperscript{44} Thus, a follicle-deplete, ovary-intact animal closely approximates the natural human progression through the events of perimenopause and the post-menopausal stage. \textsuperscript{4} vinylcyclohexene diepoxide (VCD) has been found to induce premature ovarian failure in mice and rats by accelerating the process of atresia in ovarian small pre-antral follicles.\textsuperscript{45} Previous studies in mice demonstrate that VCD-induced follicle loss can cause depletion of the smallest pre-antral follicles within 15 days of daily dosing, and complete ovarian failure within 46 days of the onset of dosing.\textsuperscript{46} As a result, the mouse retains little residual ovarian tissue. The model has been developed by treating mice with VCD to induce ovarian failure and subsequently exposing the ovary to a known carcinogen, 7, 12—dimethylbenz[a]anthracene (DMBA), to induce ovarian cancer. The VCD/DMBA model develops a variety of benign and malignant tumors.\textsuperscript{47}

Our overall goal is to develop an imaging method that can determine with certainty whether a woman’s ovaries are normal, or if ovarian cancer is developing. With such a method, high-risk women could undergo a laparoscopic diagnostic test to determine if their ovaries are healthy in order to avoid, or prolong time to, oophorectomy. In this study, we utilize second harmonic generation (SHG) microscopy to examine micron-scale collagen structure in normal, atypical, and cancerous mouse ovaries. By examining alterations in collagen structure, we may ultimately be able to identify the changes that precede ovarian cancer. Differences in SHG microscopy images of the diagnostic categories are examined by eye and quantified with numerical parameters relating to
image frequency content and second-order gray-level statistics. Further, a classification scheme is developed using a support vector machine.

Methods

Animals

All experiments were performed per NIH guidelines, and protocols were approved by the University of Arizona Institutional Animal Care and Use Committee. Female B6C3F1 mice (age 28 days, Harlan, Dublin, VA) were housed in microisolators per NIH guidelines and allowed a seven-day acclimation period before initiating the experiment. Fifty-two 28-day-old mice received intraperitoneal (IP) injections of 4-vinylcyclohexene diepoxide, 160 mg/kg/day in sesame oil, daily for 20 days, or received sesame oil vehicle only as control. Four months after the end of IP dosing, animals received a single injection of 7, 12-dimethylbenz[a]anthracene, 50 µg in 5 µl sesame oil, or 5 µl sesame oil vehicle for controls, under the bursa of the right ovary. Sterile surgical method was used to expose the ovarian bursa for sub-bursal injection. Prior to surgery animals were anesthetized by IP injection of 2% Avertin at 0.015 ml per gram body weight. The left ovary was not injected. Therefore, there were four experimental groups: both VCD and DMBA exposed, only VCD exposed, only DMBA exposed, and neither VCD nor DMBA exposed. Ovaries were harvested at 5 or 7 months after sub-bursal injection with DMBA and immediately imaged. Time from ovary excision to completion of imaging was less than one hour.

Imaging
Imaging was performed with a single-beam multiphoton microscope (TrimScope, LaVision BioTec, Bielefeld, Germany) using a Titanium:Sapphire laser light source (Chameleon Ultra2, Coherent, UK) that is coupled to the scanner unit, with a pulse width of 120 fs in the sample. The laser intensity is adjusted with an electro-optical modulator (EOM 350-80, Conoptics, USA). Simultaneous SHG and TPEF image data are recorded through non-descanning reverse detection using triple detector port equipped with Galium Arsenide (H7422A-40, Hamamatsu, Hamamatsu City, Japan) and bialkali sensors (H6780-01, Hamamatsu) and H6780-20). For this study, only the SHG image data were analyzed. The excitation wavelength was set to 780 nm, and a band pass filter FF01-377/50 (Semrock) and a dichroic mirror Di01-R405-25 x 36 (Chroma) were used to collect light from SHG. Power on the sample was set to 20 mW. Pixel dwell time was 4.61 μs and three line summing was used. Images were taken at 10 μm depth increments from the surface of the tissue to 60-100 μm depth. All images had a 400 x 400 μm field of view and contained 993 x 993 or 1021 x 1021 pixels with 14-bit gray scale resolution.

**Histology and Pathological Evaluation**

After imaging, ovaries were fixed in Bouin’s solution for 2-4 hours, transferred to 70% ethanol, dehydrated, embedded in paraffin blocks, and sectioned at 5 μm thickness. Orientation was carefully maintained from explant to imaging, fixation, paraffin embedding, and sectioning, by maintaining anatomical orientation at explant and placing the ovary face up on filter paper indicating medial-lateral and superior-inferior locations. Histology sections were taken perpendicular to the area imaged, allowing a cross-
sectional view of the imaged edge. Every 20th section was mounted and stained with hematoxylin and eosin. All histologic specimens were evaluated by a pathologist and a gynecologic oncologist with veterinary training. Any ovary with suspected tumor had additional sections immunostained with cytokeratin (anti-cytokeratin 18 antibody [E431-1] & rabbit polyclonal to wide spectrum cytokeratin, Abcam Inc., Cambridge, MA), per the manufacturer’s recommended protocol, to determine if the tumor was of epithelial origin. The specimens were diagnosed per pathologic findings into the following seven categories: normal, DMBA-effect, tubular adenoma, tubular adenoma with areas of focal dysplasia, granulosa cell tumor, Sertoli-Leydig cell tumor, or adenocarcinoma. Normal ovaries are those which contained only healthy tissue or changes consistent with a normal aging process. DMBA-effect was a benign abnormality, caused by DMBA exposure, which was characterized by epithelial cell proliferation, degenerating follicles, degenerating corpora lutea and highly active steroidogenic cells. Tubular adenoma is a benign epithelial tumor of glandular origin characterized by cells organized in tubules. The limited number of granulosa cell and Seroli-Leydig cell tumors seen precluded their inclusion in the image analysis. Adenocarcinoma is a malignant tumor arising from the epithelial cells of glandular tissue and is the most common form of ovarian cancer in women.

Analysis and Classification

Images were analyzed by eye and characteristic features were identified. On the basis of visual examination, it was expected that computation of spatial frequency content and
standard gray-level co-occurrence matrix (GLCM) parameters might capture the variations in collagen fiber thickness and periodicity seen by eye, subsequently enabling automatic classification of images into correct diagnostic groups. All images were pre-processed by resampling to 1024 x 1024 pixels using bilinear interpolation. All image processing and analysis was performed in MATLAB (R2011a, Mathworks).48

Of the fifty-two animals included in the study, a total of 92 specimens were imaged and 59 specimens were included in the analysis. Twelve specimens were not imaged due to instrument malfunction or user error. Thirty-one of the specimens were not included in the analysis because they did not contain ovary or the ovary was entirely covered by fat and/or connective tissue in the area imaged. Two other specimens were excluded from the analysis because they were the only example from a unique diagnosis (granulosa cell tumor and Sertoli-Leydig cell tumor). Images were excluded from the analysis if the average gray level was less than 1% above the noise floor of the imaging system, if they contained artifacts from fur or other debris, or if the imaged area was not ovary, as verified by histology.

After exclusion of unusable ovaries and images, the following data were available for analysis: normal (25 ovaries, 315 images), DMBA-effect (11 ovaries and 115 images), tubular adenoma (10 ovaries, 94 images), tubular adenoma with dysplasia (9 ovaries, 54 images) and adenocarcinoma (4 ovaries, 55 images).

*Fourier Analysis*
The two-dimensional discrete Fourier transform was computed for each image using the standard FFT algorithm. The images contained primarily low frequency content with some high frequency noise. In order to remove noise and analyze the lower frequency content, a frequency cut-off was determined by evaluating the fiber size by eye. The smallest collagen fiber width was approximately 6 pixels, which equates to a spatial frequency of 1/6 the maximum, so the frequency range used in the analysis was limited to the lowest 1/6 of spatial frequencies. This lower frequency range was divided into three equal-width circular bands: low, middle and high frequency bands. The power in each band was computed and normalized to the total power in the three bands. The DC value was excluded from the lowest frequency region.

Gray Level Co-Occurrence Matrix Analysis

GLCM analysis is a widely used texture analysis method developed by Haralick et al.\textsuperscript{49} The GLCM is formed by counting the number of occurrences of a gray level occurring adjacent to another gray level, at a specified pixel distance and direction. The result is a matrix with rows and columns representing gray levels, and elements containing the probability of the gray level co-occurrence. A separate matrix can be generated for each pixel separation and each direction. Symmetric GLCMs were computed using 64 gray-levels with the gray-level limits being the minimum and maximum gray-levels in the image—that is, gray-level values in each image were linearly scaled such that the highest gray-level in the image became 64 and the lowest gray-level in the image became 1. For a 1024 x 1024 pixel image, a practical upper bound on pixel separation was 50 pixels.
Separations of 1 to 50 in 1 pixel increments were used for this study. Since collagen fiber orientation was not consistent from ovary to ovary, parameters for four orientations (0, 45, 90, and 135 degrees) were computed and averaged.

From each GLCM, four parameters (contrast, correlation, energy, and homogeneity) that capture essential image characteristics were computed as follows:

Contrast: \[ \sum_{i,j} |i - j|^2 p(i, j) \]  
(1)

Correlation: \[ \sum_{i,j} \frac{(i - \mu_i)(j - \mu_j)p(i, j)}{\sigma_i \sigma_j} \]  
(2)

Energy: \[ \sum_{i,j} p(i, j)^2 \]  
(3)

Homogeneity: \[ \sum_{i,j} \frac{p(i, j)}{1 + |i - j|} \]  
(4)

where \( p(i, j) \) is the probability of gray level \( i \) occurring next to gray level \( j \),

\[ \mu_i = \sum_i \sum_j i \cdot p(i, j), \quad \mu_j = \sum_i \sum_j j \cdot p(i, j), \quad \sigma_i = \sum_i \sum_j (i - \mu_i)^2 \cdot p(i, j), \quad \text{and} \]

\[ \sigma_j = \sum_i \sum_j (j - \mu_j)^2 \cdot p(i, j). \]

All parameters have a maximum value of 1 and a minimum value of 0 or -1.\textsuperscript{48,49} There are many other parameters that can be computed from the GLCM. In order to minimize the computation time required, a few parameters with the most potential were selected.
High contrast occurs when an image has a high number of pixel pairs with large differences in gray level occurring at the specified separation and orientation. High correlation occurs in images with periodic features. Energy (or angular second moment) is highest in images with uniform gray level or uniform gray level differences at the specified separation and lower for those with more variation in gray levels. Finally, homogeneity (or inverse difference moment) is highest in images with pixels of the same or similar gray levels at the specified separation and orientation.

Combining the Fourier and GLCM parameters resulted in a total of 203 parameters to describe each single image. The first three parameters are the power in the low, middle and high frequency bands. The rest of the parameters are GLCM contrast, correlation, energy and homogeneity for 1, 2, 3, …, 50 pixel separations.

*Image Classification*

The five diagnoses were used to separate the images for classification. Carcinoma was compared to normal and carcinoma was compared to all other images (non-carcinoma). Also, benign tubular adenoma was compared to tubular adenoma with dysplasia. Image classification was performed using a support vector machine (SVM) algorithm. The SVM is a machine learning classifier for separating two classes. MATLAB default settings were used with sequential minimal optimization for finding the hyperplane and either a linear or quadratic kernel to map the data into kernel space. The classification function uses the equation,
\[ c = \sum_i a_i k(s_i, x) + b \]  \hspace{1cm} (5)

where \( s_i \) are the support vectors, \( a_i \) are the weights (slope), \( b \) is the bias (intercept), and \( k \) is a kernel function, which was chosen to be linear (dot product) or quadratic. If \( c \geq 0 \), then \( x \) is classified as a member of the first group, otherwise \( x \) is classified as a member of the second group.\(^{48}\)

Training was performed on 25\% of the data selected at random. Training was performed 100 times, with replacement of training images to the image pool before each subsequent training iteration. The performance of the classifier was determined by finding the average and standard deviation of the area under the receiver operator characteristic (ROC) curve for the 100 training sets. The ROC curve is a plot of the true positive rate versus the false positive rate (i.e., sensitivity versus 1-specificity) and is calculated using the true classes (specified by the user) and the output classes from the SVM at various bias values. A larger area under the ROC indicates better classifier performance.\(^{51, 52}\)

Sensitivity and specificity were determined by selecting the point on the ROC curve where sensitivity and specificity were approximately equal.

The minimum number of parameters for optimal binary classification of each group pair was found using sequential forward selection (SFS).\(^{53}\) For SFS the area under the ROC curve was evaluated for each of the 203 parameters using 100 training sets (all images in the training set have known diagnoses). The best single parameter was then combined with each of the remaining 202 parameters and 100 training sets were performed to find
the highest performing pair of parameters. The best two parameters were then combined
with the remaining 201 parameters and 100 training sets were performed to find the
highest performing trio of parameters. This process was repeated until the optimal set of
parameters was identified. An additional parameter was kept only if the additional
parameter increased the performance by greater than one standard deviation from the
previous performance. Differences in optimal parameters were checked for statistical
significance using a linear mixed effects model, in order to account for multiple images
from each ovary. The mixed effects model included a random intercept and a robust
sandwich estimator to estimate the covariance matrix.

In order to verify that the properly trained classifier performs better than random
guessing, the classifier was challenged with a random grouping test, in which training
was performed as above, but classes were assigned randomly instead of correctly. The
random assignments were given in the same proportion as true image classes.

Many images contained large regions without signal. Images with high and low signal
area were trained on and tested separately to see if higher signal area resulted in better
sensitivity and specificity. Images from normal and carcinoma diagnoses were placed
into the following groups based on visual inspection: images having greater than 75% of
the field of view (FOV) containing signal (19 carcinoma and 54 normal), images with
greater than 25% of the FOV containing signal (40 carcinoma and 119 normal) and
images with less than 25% of the FOV containing signal (8 carcinoma and 12 normal).
The parameters that were found to be best for normal versus carcinoma (when training on all images) were used for training and 100 iterations were performed.

Results

Images representing the general appearance of each diagnosis are shown [Fig. A.1]. Images of normal ovaries have thin, straight collagen fibers that weave in all directions around the many different sized follicles. Images from DMBA-effect ovaries have various size fibers with small voids in some areas. Tubular adenoma ovary images have primarily thin collagen fibers with distinctive fuzzy dots. Images from ovaries with tubular adenoma and tubular adenoma with dysplasia are similar, but more variation in collagen fiber thickness can be seen in tubular adenoma with dysplasia. The carcinoma ovary images tend to have thick, wavy collagen fibers that are ordered in the same direction, close together and in thick bands not covering the entire field of view.

The sequential forward selection resulted in selection of two or three image analysis parameters for each binary classification. Using more than three parameters never resulted in improved performance. Carcinoma was best differentiated from normal alone, and from all other diagnoses combined, using two parameters: power in the highest frequency region (PHF) and GLCM energy with 38 pixel separation. Tubular adenoma was best differentiated from tubular adenoma with dysplasia using three parameters: GLCM contrast at 17 and 25 pixel separation and GLCM energy at 22 pixel separation. The average values of these five parameters are shown in Figures A.2 and A.3. There is a statistically significant difference between carcinoma and all other diagnoses for PHF (p
There is a statistically significant difference between cancer and all other diagnoses, except tubular adenoma with dysplasia, for GLCM energy with 38 pixel separation (p ≤ 0.0025) [Fig. A.2]. There is a statistically significant difference between tubular adenoma and tubular adenoma with dysplasia for GLCM contrast at 17 and 25 pixel separation and GLCM energy at 22 pixel separation (p ≤ 0.0015) [Fig. A.3].

Visualizing these data in another manner, Figure A.4 shows a plot of PHF versus GLCM energy at 38 pixel separation for carcinoma and normal diagnoses. Carcinoma values appear to fall on the outer border of the normal values. Figure A.5 shows a plot of GLCM contrast at 17 and 25 pixel separations for tubular adenoma and tubular adenoma with dysplasia diagnoses. The tubular adenoma with dysplasia values appear to fall on a line with similar slope but higher intercept than tubular adenoma.

The area under the ROC curve (AUC) was always greater than 0.78 for correct class and less than 0.61 for random class, showing that all training groups that were trained with the correct class performed better than training groups that were trained with random class assignments (25% of data in training, 75% testing, 100 iterations). Also, quadratic kernel resulted in similar performance as compared to linear kernel (AUC ≈ 0.83 for linear and AUC ≈ 0.81 for quadratic) for carcinoma versus normal and carcinoma versus non-carcinoma.

When training on 25% and testing on the remaining 75%, the classifier showed better than 74% average sensitivity and specificity results for all groups. The quadratic kernel resulted in higher sensitivity and specificity as compared to the linear kernel for
carcinoma versus normal (77.8% and 79.3%, compared to 75.4% and 76.1%, respectively). The same result was seen for carcinoma versus non-carcinoma (81.2% and 80.0%, compared to 76.6% and 75.4%). The highest sensitivity and specificity achieved for each group are shown in Table A.1.

For carcinoma versus normal, images with signal in less than 25% of the FOV had low sensitivity and specificity. Using images with signal in greater than 25% of the FOV produced sensitivity and specificity slightly better than using all images. Using images with signal in greater than 75% of the FOV produced the highest sensitivity and specificity [Table A.2].

Discussion

The VCD/DMBA-treated animal model successfully led to ovarian adenocarcinoma, the most commonly occurring ovarian malignancy in women. Therefore, the collagen structure changes seen in this model may be similar to structural changes seen in human ovarian cancer. Unfortunately, the incidence of adenocarcinoma was only 35% in the VCD/DMBA group (and 0% in the control groups). Thus, the number of carcinoma specimens was very small compared to the number of specimens from other diagnoses. Combined with relatively large variation in the appearance of carcinoma regions, the analysis results presented here must be considered preliminary.

Both VCD and DMBA caused atrophy of ovarian tissue and occasionally adhesions, making the ovaries difficult to find and separate from fat and other tissue. This difficulty resulted in exclusion of many samples that were histologically confirmed as non-ovarian
tissue in the area imaged. Also, VCD and DMBA caused effects in the ovary that are not common in women. VCD caused the development of benign tubular adenomas. Tubular adenomas are rare in the human ovary so the findings related to this, although possibly useful in a very small population, would not directly translate to changes seen in women at high risk for adenocarcinoma. Occasionally the tubular adenomas also developed focal areas of dysplasia. Since dysplasia often precedes cancer, identification of collagen changes due to dysplasia would be very useful for detecting pre-cancerous changes in human ovary, but it is unclear if the collagen morphology changes accompanying tubular adenoma with dysplasia would be the same as collagen morphology changes occurring during dysplasia in the absence of tubular adenoma.

DMBA caused a condition which was labeled “DMBA-effect.” The DMBA-effect included changes associated with highly active steroidogenic cells, degenerating follicles, degenerating corpora lutea and a proliferative epithelial layer. The entire effect is not seen in humans but proliferation of the epithelial layer is a risk factor for ovarian cancer, so the collagen changes that were seen in the DMBA-effect may translate to humans if the change in collagen morphology is related to epithelial proliferation. Average parameter values for DMBA-effect images frequently fell between values for normal and carcinoma, suggesting the possibility that collagen changes may be due to proliferation of the epithelial layer, however the differences in parameter values for DMBA-effect and normal were not statistically significant.
Training results revealed that power in the highest frequency band and GLCM energy with 38 pixel separation were the most useful parameters for separation of both carcinoma versus normal and carcinoma versus non-carcinoma images. It is expected that the frequency content of the images would vary from group to group because the collagen fiber width is visually different. In the carcinoma images, collagen fibers appear to be much thicker than the collagen fibers in the tubular adenoma or normal images, which will translate to lower relative power in the high frequency region. It is also expected that GLCM energy would change for different collagen morphologies because the collagen width and spacing affects the transition of gray levels across the image. Normal has lower average energy at 38 pixel separation because the fibers are thinner, causing more variation in gray levels when moving across the image.

The increase in performance seen for carcinoma versus non-carcinoma over carcinoma versus normal is likely due to carcinoma having greater separation from other diagnoses than from normal. For example, carcinoma and tubular adenoma with dysplasia have a larger separation in the average value for power in the high frequency region than carcinoma and normal. Difficulty in distinguishing the carcinoma and normal categories is likely due to the wide variation in collagen structure within normal ovaries, leading to a variation in computed image features. Normal ovaries have considerable image variation because the collagen structure depends on the structures it surrounds—stromal cells, follicles, or scar tissue from a new corpus luteum will all look different.
For simplicity, we initially intended to use only a linear kernel for classification. However, due to the parabolic shape of the plot of the parameters [Fig. A.4] for carcinoma and normal, a quadratic kernel was also tested. Training performance of the quadratic kernel was slightly lower (AUC lower) than the linear kernel, but the quadratic kernel gave better sensitivity and specificity results for both carcinoma versus normal and carcinoma versus non-carcinoma. There was an increase in sensitivity and specificity despite a decrease in AUC because the portion of the ROC curve near equal sensitivity and specificity for the quadratic kernel was above the ROC curve for the linear kernel at the selected point. If another point on the ROC curve was selected, the sensitivity and specificity may be greater for the linear kernel than for the quadratic kernel. These results suggest that the ideal line for separation is neither linear nor quadratic.

Because of varying surface topology, clefts, and invaginations in the ovaries, as well as variations in ovary composition, there were frequently large portions of the image FOV that did not contain signal. Sensitivity and specificity results were similar to random when only images with less than 25% of the FOV containing signal were used. The sensitivity and specificity results were improved when only images containing signal in greater than 25% of the FOV (rather than all images) were analyzed. Restricting analysis to only images with greater than 75% of the FOV containing signal resulted in incremental improvement, but also required the exclusion of many images. These results indicate that images used for detection of cancer require signal in at least ¼ of the FOV. In this study, the presence of images with a small percentage of the FOV containing signal was mainly due to the small radius of curvature of the mouse ovaries, which, when
combined with the water immersion objective, precluded a flat field of view. The radius of curvature of a human ovary would be much larger and a clinical implementation of the system would likely be a contact probe, allowing the physician to obtain an image of a flat surface. When imaging a flat surface, there is likely to be signal in the entire FOV, reducing the number of images that would need to be excluded from the analysis.

Tubular adenoma and tubular adenoma with dysplasia were separated with the largest sensitivity and specificity of the three groups tested. For tubular adenoma versus tubular adenoma with dysplasia, training results showed that the most useful parameters were GLCM contrast at 17 and 25 pixel separation and GLCM energy at 22 pixel separation. The excellent performance when using energy and contrast at different pixel separations is likely related to the periodicity of fiber spacing in the images, which was generally between 5 and 50 pixels. These results indicate that tubular adenoma images contain a pattern that repeats at approximately 22 pixel separation and larger differences in gray levels at 17 and 25 pixel separations than tubular adenoma with dysplasia. Images of tubular adenoma and tubular adenoma with dysplasia had a very distinct visual appearance, with the presence of dots intermixed with numerous thin fibers. It is unclear what tissue constituent is responsible for these dots and more investigation is required to find the cause of this signal. Clinically, distinguishing a benign condition from dysplasia would be useful, although as stated, the incidence of tubular adenoma in women is small.

The high sensitivity to dysplastic changes is particularly exciting because the histologically verified region of dysplasia was typically deep in the ovary, not in the
superficial volume imaged. This finding suggests that there may be a field effect influencing the surrounding collagen structure for hundreds of micrometers. A field effect has been noted in light scattering spectroscopy of the colon.\textsuperscript{54} However, as the number of dysplastic ovaries in this study was small, a firm conclusion cannot be drawn.

Classifier performance for every group was higher with true diagnosis assignments than with random diagnosis assignments. This result proves that the classifier is able to separate better than random chance.

Twenty to fifty percent of BRCA-positive (high risk) patients develop ovarian cancer.\textsuperscript{55} If 1000 BRCA-positive patients underwent prophylactic oophorectomy, 500-800 of these procedures would have been extraneous, causing unnecessary morbidity and mortality. If 1000 patients were tested before oophorectomy using a diagnostic test with 80% sensitivity and specificity, as we have shown, then only 100-160 would be unnecessary oophorectomies. The trade-off is that 100-160 women with cancer would not be caught by the test. By changing the bias value on the ROC curve, 100% sensitivity and 51% specificity can be selected. Using the corresponding curve for separation, the diagnostic test would successfully detect all cancers and result in unnecessary oophorectomy of 245-392 patients. As compared to the current method of prophylactic ovary removal, this diagnostic test would cut the number of unnecessary oophorectomies in half, greatly reducing unnecessary morbidity and mortality.

The analysis and classification methods used here provided a simple way to separate two classes at a time. A more sophisticated classifier would enable multiple diagnoses, and
would incorporate data from a full stack, or multiple stacks of images, to capture the variation in ovary morphology. The differences in collagen structure between categories are often very subtle, and there is significant variation within a group, particularly in the case of normal. Tumors are often much more uniform throughout—this is seen most obviously in the tubular adenoma, which look very similar from slice to slice and ovary to ovary. Additionally, the dynamic changes in collagen structure may make the same diagnosis appear different depending on the extent of disease. In the future, we plan to examine whether combining SHG images with two-photon excited fluorescence images and/or optical coherence tomography images can improve classifier performance. We also plan to analyze images obtained in vivo at multiple time points during an animal’s lifetime in order to better evaluate changes during early disease development. The in vivo imaging series will allow us to select ovaries with disease at the end of the study and look back in time to determine changes in collagen structure during early disease. With better understanding of early disease, we hope to develop an optical diagnostic test for ovarian cancer to use in women who are candidates for prophylactic oophorectomy. The ideal diagnostic system would be implemented minimally invasively using a micro-endoscope, like a falloposcope, that could image both the fallopian tubes and ovaries. Such a test could potentially reduce the number of unnecessary salpingo-oophorectomies in high risk women and thus improve their quality of life.

Acknowledgements: This study was sponsored in part by National Institute of Health, National Cancer Institute research grant R01 CA119200 and the University of Arizona Cancer Center Support Grant (CCSG - CA023074).
References


Fig. A.1 Images from three ovaries of each diagnosis: normal (A-C), DMBA-effect (D-F), tubular adenoma (G-I), tubular adenoma with dysplasia (J-L), and carcinoma (M-O).
Fig. A.2 Average parameter values for parameters used to separate carcinoma from normal and carcinoma from non-carcinoma. TA=tubular adenoma. TD=tubular adenoma with dysplasia. Only positive standard deviations are shown.

Fig. A.3 Average parameter values for parameters used to separate tubular adenoma (TA) from tubular adenoma with dysplasia (TD). Only positive standard deviations are shown.
Fig. A.4 Plot of power in the high frequency region versus GLCM energy for carcinoma and normal diagnoses.

Fig. A.5 Plot of contrast with 17 pixel separation vs. contrast with 25 pixel separation for tubular adenoma (TA) and tubular adenoma with dysplasia (TD).
Table A.1 Testing results for 100 iterations (standard deviation in parentheses). TA=tubular adenoma. TD=tubular adenoma with dysplasia.

<table>
<thead>
<tr>
<th>Groups Tested</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma vs. Normal</td>
<td>77.8 (11.3)</td>
<td>79.2 (6.8)</td>
</tr>
<tr>
<td>Carcinoma vs. Non-Carcinoma</td>
<td>81.2 (11.1)</td>
<td>80.0 (5.0)</td>
</tr>
<tr>
<td>TA vs. TD</td>
<td>80.2 (3.8)</td>
<td>82.7 (4.6)</td>
</tr>
</tbody>
</table>

Table A.2. Testing results for carcinoma versus normal (standard deviation in parentheses).

<table>
<thead>
<tr>
<th>Signal Area</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;25%</td>
<td>62.0 (32.4)</td>
<td>66.7 (14.4)</td>
<td>77.8 (11.3)</td>
<td>79.2 (6.8)</td>
</tr>
<tr>
<td>&gt;25%</td>
<td>79.5 (15.0)</td>
<td>81.3 (9.7)</td>
<td>81.5 (12.7)</td>
<td>81.1 (9.7)</td>
</tr>
<tr>
<td>&gt;75%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A.2 File Locations

Files associated with SHG paper: \MSPACMAN\Data
(E)\DroboData\JenWatson\Authored Papers\JBO_SHG_ex_vivo

Image naming scheme for raw data: m (for mouse) followed by O (for ovary), followed by the two or three digit mouse ID#, followed by R or L (for right or left), followed by the dosing group (CON, VCD, DMBA), followed by the date in yymmdd format, followed by MP (for multiphoton) or OCT. Multiphoton channels SHG (SA), NADH (NA), FAD (FD) are labeled with abbreviations or labeled HS1, HS2, PMT6, respectively.

Raw OCT data (in folders by date that images were acquired) are located in folder: \MSPACMAN\Data (E)\DroboData\Mouse_Study_2010Exvivo\OCT_exvivo

Raw multiphoton data (including SHG and TPEF .msr files and .tiff exports) are located in folders labeled with the date the multiphoton data were acquired are located in: \MSPACMAN\Data (E)\DroboData\Mouse_Study_2010Exvivo\MP_exvivo

Subsets of SHG image stacks used for analysis (have had images with poor signal and artifacts removed) are located in folders labeled by diagnosis (files named by mouse ID# and R or L):
\MSPACMAN\Data(E)\DroboData\Mouse_Study_2010Exvivo\Image_analysis\BrewDia gAnaly\NewDiagImg\Image_stacks
Additional subsets of images with small field of view containing signal:
\MSPACMAN\Data(E)\DroboData\Mouse_Study_2010Exvivo\Image_analysis\BrewDia
gAnaly\BrewSHG\lowsig

Additional subsets of images with large field of view containing signal:
\MSPACMAN\Data(E)\DroboData\Mouse_Study_2010Exvivo\Image_analysis\BrewDia
gAnaly\BrewSHG\highsig

Fourier and texture analysis files: \MSPACMAN\Data
(E)\DroboData\Mouse_Study_2010Exvivo\Image_analysis\Fourier Analysis

Older texture analysis located in: \MSPACMAN\Data
(E)\DroboData\Mouse_Study_2010Exvivo\Image_analysis\GLCM

Sequential forward search for 1, 2, …, parameters named “FSS_nparameters” in folder:
\MSPACMAN\Data(E)\DroboData\Mouse_Study_2010Exvivo\Image_analysis\BrewDia
gAnaly\BrewSHG\files_after_review_Jan2012
APPENDIX B: TWO-PHOTON EXCITED FLUORESCENCE IMAGING OF ENDOGENOUS CONTRAST IN A MOUSE MODEL OF OVARIAN CANCER

B.1 Published Manuscript: Two-photon excited fluorescence imaging of endogenous contrast in a mouse model of ovarian cancer

The following manuscript was published in the 45(3) 2013 issue of Lasers in Surgery and Medicine (first published online January 2013).
Title: Two-photon excited fluorescence imaging of endogenous contrast in a mouse model of ovarian cancer

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Funding: National Institutes of Health, National Cancer Institute research grant R01 CA119200 and the University of Arizona Cancer Center Support Grant (CCSG - CA023074).

Key words: aging, carcinoma, lipofuscin, lipoprotein, multiphoton, microscopy, NADH

Abstract:

Background and Objective: Ovarian cancer has an extremely high mortality rate resulting from poor understanding of the disease. In order to aid understanding of disease etiology and progression, we identify the endogenous fluorophores present in a mouse model of ovarian cancer and describe changes in fluorophore abundance and distribution with age and disease.

Study design/Materials and Methods: A mouse model of ovarian cancer was created by dosing with 4-vinylecyclohexene diepoxide, which induces follicular apoptosis (simulating menopause), and 7, 12-dimethylbenz[a]anthracene, a known carcinogen. Imaging of ovarian tissue was completed ex vivo with a multiphoton microscope using excitation wavelength of 780 nm and emission collection from 405-505 nm. Two-photon
excited fluorescence images and corresponding histologic sections with selective stains were used to identify endogenous fluorophores.

**Results:** The majority of collected fluorescence emission was attributed to NADH and lipofuscin, with additional contributions from collagen and elastin. Dim cellular fluorescence from NADH did not show observable changes with age. Changes in ovarian morphology with disease development frequently caused increased fluorescence contributions from collagen and adipose tissue-associated NADH. Lipofuscin fluorescence was much brighter than NADH fluorescence and increased as a function of both age and disease.

**Conclusions:** Our finding of NADH fluorescence patterns similar to that seen previously in human ovary, combined with the observation of lipofuscin accumulation with age and disease also seen in human organs, suggests that the findings from this model may be relevant to human ovarian disease. Increased lipofuscin fluorescence might be used as an indicator of disease in the ovary and this finding warrants further study.

**Introduction:**

Ovarian cancer is a devastating disease, with more than 225,000 new cases diagnosed and over 140,000 deaths each year worldwide [1]. When diagnosed at early stages the survival rate is high. However, due to lack of specific symptoms or an effective screening test, few women present with early stage disease. This situation leads to high mortality, as well as a lack of understanding of the development of ovarian cancer. The limited data available on women necessitates the use of an animal model to discover information
about early cancerous changes. Discoveries in animal models can be translated to humans, to enable development of early diagnosis and treatment strategies.

A variety of ovarian tumor types can develop in women but adenocarcinoma is the most frequently occurring ovarian disease leading to death in women. For this reason, we use an animal model that develops adenocarcinoma, among other tumor types, through dosing with 4-vinylcyclohexene diepoxide (VCD) and 7, 12-dimethylbenz[a]anthracene (DMBA). Dosing with VCD causes ovarian failure, simulating menopause. Creating ovarian failure in the mouse allows us to better approximate the hormonal environment in women who develop ovarian cancer, as they are most frequently peri- or post-menopausal. Further, by using an animal model that develops multiple tumor types, we can compare findings among tumors to identify if the changes observed are present among all diseased tissues or are specific to tumor cell origin or benign or malignant disease.

Optical imaging has shown great promise for detection of ovarian cancer and evaluation of disease progression, due to high resolution and sensitivity to tissue alterations. Fluorescence imaging and spectroscopy are particularly useful for early detection of disease because of the technique’s high sensitivity to endogenous or exogenous fluorophores, and flexibility in scale from wide field to microscopic. Tissue fluorophores absorb incident light and subsequently emit light, usually of a longer wavelength. The wavelengths and intensity of the remitted light are related to the quantity, type, and distribution of fluorophores present. Several types of fluorescence-based instruments
have been used to study ovarian tissue, including point-sampling fluorescence spectroscopy [2-6], surface imaging fluorescence microscopy [7-8], depth-resolved confocal microscopy [2,9,10], and two-photon excited fluorescence (TPEF) microscopy [11-13].

George et al. used point fluorescence spectroscopy with excitation over 270-550 nm and emission collected over 290-700 nm to show differences in the endogenous fluorescence spectra between malignant and non-malignant human ovarian tissue [5]. Using laser-induced fluorescence spectroscopy, Hariri et al. observed spectral differences in cyclic, acyclic and neoplastic rat ovaries [4]. Adur et al. used TPEF to visualize cells in fixed, H&E stained sections of ovarian epithelial cancer [12]. Kirkpatrick et al. used TPEF to study changes in cell metabolism with disease and demonstrated that normal low-risk human ovaries have a lower redox ratio (calculated from the intensity of wavelengths associated with the fluorescence emission of metabolic co-factors nicotinamide adenine dinucleotide [NADH] and flavin adenine dinucleotide [FAD]) than human ovaries with cancer [13].

TPEF has the advantage of high resolution, depth resolved imaging, with an imaging depth greater than confocal imaging. The greater depth of imaging is achieved by using near infrared short-pulsed light. The longer wavelengths penetrate deeper than ultraviolet or visible light, which are typically used in single photon techniques such as confocal microscopy and surface fluorescence imaging. Additionally, at near-infrared wavelengths there is minimal absorption and tissue damage. The short pulses of light and highly
focused beams create a high instantaneous power density, resulting in a high probability of two photons being simultaneously absorbed by a fluorophore, followed by emission of one photon at a shorter wavelength than the incident light. The two-photon phenomenon is only probable at the focus of the beam, strongly suppressing out-of-focus signal and enabling deeper imaging.

TPEF allows for utilization of endogenous fluorescence, eliminating the possibility of toxicity or other side effects from exogenous contrast agents, and simplifying the imaging procedure. Endogenous fluorophores in the body include proteins containing aromatic amino acids (tryptophan, tyrosine, phenylalanine), metabolic co-factors such as NADH and FAD, structural proteins such as collagen and elastin, and a variety of other molecules including vitamins and lipofuscins [14, 15].

In this study, we utilize TPEF for high resolution, deep visualization of endogenous fluorescence in the mouse ovary. Acquiring better understanding of the relationship between fluorescence and tumor development in this mouse model may lead to improved methods for diagnosis and treatment of ovarian cancer in women. We hope to validate the usefulness of TPEF imaging for identifying disease related changes so that we may pursue in vivo imaging studies for disease detection in the future. We identify endogenous fluorophores present in the mouse ovary, and describe changes that occur with age and disease. Further, we discuss the potential translatability of these findings to women.

Methods:

Animals
All experiments were performed per NIH guidelines, and protocols were approved by the University of Arizona Institutional Animal Care and Use Committee. Female B6C3F1 mice (age 21 days, Harlan, Dublin, VA) were housed in microisolators and allowed a seven-day acclimation period before initiating the experiment. Fifty-two 28-day-old mice received intraperitoneal (IP) injections of 4-vinylcyclohexene diepoxide, 160 mg/kg/day in sesame oil, daily for 20 days, or received sesame oil vehicle only as control. Four months after the end of IP dosing, animals received a single injection of 7, 12-dimethylbenz[a]anthracene, 50 µg in 5-µl sesame oil, or 5-µl sesame oil vehicle for controls, under the bursa of the right ovary. Animals were anesthetized by IP injection of 2% Avertin at 0.015 ml per gram body weight and sterile surgical method was used to expose the ovarian bursa for sub-bursal injection. The left ovary was not injected. Therefore, there were four experimental groups: VCD and DMBA exposed (VCD/DMBA), VCD and vehicle injection (CON/VCD), DMBA and vehicle injection (CON/DMBA), or vehicle injection only (CON/CON). Mice were sacrificed at 5, 7 or 9 months after sub-bursal injection with DMBA (age 11, 13, or 15 months, respectively). Ovaries were harvested immediately after euthanasia, rinsed with saline and were imaged using TPEF within one hour of excision.

TPEF Imaging

Imaging was performed with a single-beam multiphoton microscope (TrimScope, LaVision BioTec, Bielefeld, Germany) using a Titanium:Sapphire laser light source (Chameleon Ultra2, Coherent, UK) that was coupled to the scanner unit, with a pulse
width of 120 fs at the sample. The laser intensity was adjusted with an electro-optical 
modulator (EOM 350-80, Conoptics, USA). A water-immersion, 20X magnification, 0.95 
NA objective (Olympus) was used for imaging. However, rather than water, a more 
viscous surgical lubricant (Surgilube) was used for index matching with the objective. 
Simultaneous SHG and TPEF image data were recorded through non-descanning reverse 
detection using triple detector port equipped with Galium Arsenide (H7422A-40, 
Hamamatsu, Hamamatsu City, Japan) sensors. For this study, only the TPEF image data 
were analyzed. The excitation wavelength was set to 780 nm, and a band pass filter 
HQ450/100M-2p-25 (Chroma) and a dichroic mirror 505dcxr (Chroma) were used to 
collect light from TPEF. Power on the sample was set to 20 mW. Pixel dwell time was 
4.61 µs and three-line summing was used. Images were taken at 10 µm depth increments 
from the surface of the tissue to 60-200 µm depth, resulting in 6 to 20 images per ovarian 
tissue specimen. Thus, TPEF imaging was completed in less than three minutes per 
specimen. All images had a 400 x 400 µm field of view and contained 993 x 993 or 1021 
x 1021 pixels with 14-bit gray scale resolution.

Histology and Pathological Evaluation

After imaging, ovaries were fixed in Bouin’s solution for 2-4 hours, transferred to 70% 
ethanol, dehydrated, embedded in paraffin blocks, and sectioned at 5 µm thickness. 
Orientation was carefully maintained on filter paper so that the imaged region of the 
ovary was known. Multiple sections were mounted on glass microscope slides and 
covered with a layer of paraffin for preservation until sections were used for various
staining protocols. For visual analysis and display purposes, great care was taken to obtain histological sections from similar locations on the same specimen to use for comparison of TPEF images and stained sections. However, histologic specimens were taken perpendicular to the imaged plane; thus, exact corresponding sections were not possible.

Every 20\textsuperscript{th} section was stained with hematoxylin and eosin for standard structure-based pathology evaluation. Any ovary with suspected tumor had 2-3 additional sections immunostained with cytokeratin (anti-cytokeratin 18 antibody [E431-1] & rabbit polyclonal to wide spectrum cytokeratin, Abcam Inc., Cambridge, MA), per the manufacturer’s recommended protocol, to determine if the tumor was of epithelial origin.

All histologic specimens were evaluated by a pathologist and a gynecologic oncologist. The specimens were diagnosed per pathologic findings into the following six categories: normal, DMBA-effect, tubular adenoma, tubular adenoma with areas of focal dysplasia, sex cord-gonadal stromal tumor, or adenocarcinoma. Normal ovaries were those that contained only healthy tissue or changes consistent with a normal aging process. DMBA-effect was a benign abnormality, caused by DMBA exposure, which was characterized by epithelial cell proliferation, degenerating follicles, degenerating corpora lutea and highly active steroidogenic cells. Tubular adenoma was a benign epithelial tumor of glandular origin characterized by cells organized in tubules. The sex cord-gonadal stromal tumors consisted of a granulosa cell tumor and a sertoli leydig tumor, which are both rare tumors
in humans. Adenocarcinoma is a malignancy arising from the epithelial cells of the ovary and is the most common form of ovarian malignancy in humans.

Additional sections were stained and imaged to elucidate the origin of fluorescence signal. Previous studies have shown lipopigment fluorescence located in unstained cells throughout many tissues, including the ovarian stroma [16-18]. Furthermore, after staining with Sudan Black B, lipopigment fluorescence is blocked [19, 20]. Therefore, randomly selected unstained specimens from normal and other diagnoses at each time point were imaged with a fluorescence microscope, stained with Sudan Black B and then re-imaged with the fluorescence microscope.

For Sudan Black B staining, sections were dewaxed, rehydrated, stained with Sudan Black B (Sigma-Aldrich) overnight at room temperature, differentiated in 70% alcohol to remove background stain and counterstained with methyl green (Sigma-Aldrich), dehydrated and mounted in Permount (Fisher Scientific). Sudan Black B is a dye that stains lipids non-specifically but dissolves readily in phospholipids. Under this staining protocol, positive staining for lipid, neutral fat, phospholipid, or lipopigment is black, nuclei stain green, and red blood cells stain black.

Lipofuscins have been known to excite from the UV to the red and emit from the blue to the red [16-18, 21]. It was previously shown that lipofuscin was the dominant signal in normal retinal pigment epithelium (RPE) when excited with 633, 568 and 488 nm light [21]. When excitation wavelengths less than 488 were used, strong autofluorescence was seen from other fluorophores in the RPE [21]. In order to see lipofuscin as the dominant
signal we chose to use green light for excitation. Fluorescence imaging was completed with an Olympus IX70 fluorescence microscope. The light source was a mercury arc lamp with a 540/25 nm band pass excitation filter, and a 605/55 nm band pass emission filter was used for collection. Integration time was adjusted to obtain a measurable signal and Sudan Black B stained sections had an integration time twice that of unstained sections.

To further narrow the possible lipids resulting in fluorescence and verify the presence of the age-related lipopigment, lipofuscin, additional sections from each adenocarcinoma specimen and randomly selected specimens from normal and other diagnoses at each time point were stained with carbol fuchsin according to modified Zeihl-Neelsen technique. This method has been used to stain acid-fast lipofuscin in many tissues including the ovary [17, 18, 22]. Sections were dewaxed, rehydrated, stained with carbol fuchsin (Sigma-Aldrich) overnight at room temperature, differentiated in acid alcohol (1% HCL in 70% ethanol) to remove background staining, counterstained with .25% methylene blue (Sigma-Aldrich) in 1% acetic acid, dehydrated and mounted in Permount (Fisher Scientific). Under this staining protocol, positive staining for lipofuscin is deep magenta/purple, nuclei stain blue, and red blood cells stain pink. To confirm co-localization of bright fluorescence with lipofuscin, a section of normal tissue was imaged as described above (for Sudan Black B stained sections) with an Olympus IX70 fluorescence microscope before and after carbol fuchsin staining.
Each adenocarcinoma specimen and randomly selected normal specimens from each time point were also immunostained for F4/80 antibody to ovarian macrophages. Sections were dewaxed and rehydrated, endogenous peroxidase activity was blocked by immersion in 0.3% hydrogen peroxide in methanol for 15 minutes, then sections were rinsed. Antigen retrieval was performed by immersing sections in 10mM sodium citrate buffer (pH6) at 95-100°C for 20 minutes. Sections were rinsed and blocked for non-specific binding with 10% goat serum for 15 minutes at room temperature and rinsed again. Sections were incubated overnight at 4°C in primary antibody, rat anti-mouse-F4/80 (Abd Serotec). Sections were rinsed and incubated 60 minutes at room temperature with secondary antibody, goat anti-rat IgG biotinylated (Vector Labs) and rinsed again. Sections were incubated with streptavidin (Dako) 10 minutes at room temperature, rinsed and stained with DAB (Dako) for approximately 1 minute. Sections were rinsed, counter-stained with hematoxylin, dehydrated, and mounted. Under this staining protocol, cell nuclei are purple and positive staining for F4/80 macrophage is dark brown.

Quantitative Image analysis:

Prior to analysis, each image was resized and converted to binary in ImageJ (NIH). TPEF images were resized from 993 x 993 pixels or 1021 x 1021 pixels to 1024 x 1024 pixels using bilinear interpolation. Only images with signal in at least 25% of the field of view (by eye) were analyzed. Images were converted to binary by a blinded observer using the “MaxEntropy” threshold, such that the brightest regions were made white and all other
pixels were made black. The max entropy threshold uses probability distributions of the foreground and background gray levels to determine the optimal threshold [23].

Analysis of the binary images was performed in Matlab (Mathworks). Each region of white pixels was labeled using connected component labeling with 8-connectivity. Visual inspection of these images showed bright fluorescence occurring either as small punctate dots or as large (cellular or multi-cellular-sized), round-to-oval-shaped blobs. Further inspection revealed copious punctate fluorescence 6 or less pixels in diameter or less, and another, less numerous, group of fluorescent regions greater than 6 pixels in diameter. We translated the diameter to an area of $6^2$ pixels, or 36 pixels (corresponding to approximately $5.5 \, \mu m^2$ area). Analysis of the connected-component area-count histograms confirmed this visual observation. Based on bimodality of the connected-component area-count histogram, white regions were separated into two groups: those containing 36-pixel-area or less and those containing greater than 36-pixel-area. For each image, three parameters were recorded: the number of regions less than or equal to 36-pixel-area and both number of regions greater than 36-pixel-area and the average area (in pixels) of regions with greater than 36-pixel-area.

Statistics:

Statistical analysis for TPEF fluorescent regions was completed using a linear mixed effects model with random intercepts and a sandwich standard error estimator for each diagnosis in order to account for both within animal variation and between animal
variations. In addition, Tukey-Kramer p-value adjustment was done to account for multiple comparisons. Differences were considered statistically significant for p<0.05.

Results:

Animals

A total of 88 specimens from 52 animals were imaged. Sixteen specimens were not imaged due to unexpected death of the animal, instrument malfunction or user error. Of the 88 specimens imaged, 57 specimens were included in the analysis. The remaining specimens were not included in the analysis because they did not contain ovary or the ovary was entirely covered by fat and/or connective tissue in the area imaged, as confirmed by histology. Five-hundred-seventy-four images from the 57 specimens were included in the TPEF analysis. This number included 269 normal, 57 carcinoma, 95 DMBA effect, 63 tubular adenoma, 48 tubular adenoma with dysplasia and 42 sex-cord tumor images.

All CON/CON animals imaged had normal ovaries. CON/DMBA dosed animals were normal or developed DMBA effect or granulosa cell tumor in the non-DMBA dosed ovary. In the DMBA-dosed ovary, CON/DMBA animals developed DMBA effect. All CON/VCD animals developed tubular adenomas (with or without dysplasia). All VCD/DMBA animals developed adenocarcinoma in the DMBA-dosed ovary and tubular adenoma (with or without dysplasia) or sex cord-gonadal stromal tumor in the non-DMBA dosed ovary [Marion SL et al., in press, Compar Med]. The number of ovaries and images with each diagnosis at each age are shown in Table B.1.
Origin of TPEF

A number of endogenous fluorophores, including NADH, FAD, collagen, elastin and lipoproteins, experience two-photon excitation using laser wavelengths near 780 nm [12,13,24-26]. Furthermore, many of these fluorophores have emission in the range of the TPEF light collected (405-505 nm). NADH and FAD, cofactors used in metabolic processes in the mitochondria, have peak emission in the range of 440-460 nm and 515-520 nm, respectively [14, 15, 27]. It has been noted that NADH fluorescence emission maximum is shifted to 520 nm in adipose tissue [27]. Collagen and elastin are present in blood vessels and collagen is present throughout the tissue, providing support to the structure of the ovary. Both fluorophores have peak emission at 390-400 nm [14, 15, 27]. Collagen and elastin are easily distinguished from other fluorophores in the ovary due to their fibrillar structure [24]. Lipopigments associated with lipid oxidation, such as ceroid and lipofuscin, are generally found as cytoplasmic granules and have broad emission, including peaks in the 430-460 nm and 540-640 nm range [14]. Based on excitation and emission wavelengths, as well as relative abundance of fluorophores in the ovary, we expect the majority of our collected signal to originate from NADH and lipopigments.

TPEF images in this study show a broad range of fluorescence intensities, including dim and bright levels of fluorescence with different morphologies. The origin of the fluorescence signal was determined based on comparison of TPEF images with corresponding histology. Dim fluorescence was observed in regions corresponding to the cytoplasm of cells seen in H&E stained histological sections. The dim fluorescence is
most easily seen in luteal cells in corpora lutea and granulosa cells surrounding developing follicles. In these cells, the intracellular fluorescence surrounds a dark spot with minimal fluorescence intensity, corresponding to cell nuclei in the H&E stained section (Fig. B.1). Dim cellular fluorescence corresponds to NADH [13, 24-26]. Similar dim fluorescence is seen in adipose cells, which are visible in some adenocarcinoma specimens (Fig. B.2). The fluorescence in adipose cells is anticipated to be mainly from NADH [25, 27].

Additional dim fluorescence was seen in thin linear bands throughout the tissue. This morphology was frequently seen in TPEF images of carcinoma specimens. Corresponding H&E stained sections show an abundance of bands of collagenous connective tissue in the tumor. Therefore, this fluorescence is likely from collagen (Fig. B.3). Similar linear fluorescence was also seen in ring-like structures in normal ovary. Comparison of these images to H&E shows that the ring-like structures correspond to collagen and elastin in blood vessels in the ovary (Fig. B.4) [24].

Isolated, bright punctate fluorescence, 1-3 µm in diameter, was seen throughout TPEF images of ovarian interstitial tissue in all diagnoses. However, the punctate material was not identifiable in H&E stained sections. Others have also noted some extremely bright punctate fluorescence in ovarian tissue [13, 24] and have hypothesized that this fluorescence comes from fluorescent lipopigments such as lipofuscin. Further, similar bright fluorescence has been noted in cell culture and has been attributed to lipofuscin fluorescence through identification of its lysosomal localization [26].
In addition to the punctate fluorescence, some TPEF images show large regions (larger than 36-pixel area) with moderate-to-extremely bright fluorescence. Sometimes, these large fluorescent regions also contain brighter, punctate fluorescence. The bright fluorescence from the larger, cellular sized, regions appeared to correspond with the cytoplasm of large foamy cells, seen in the interstitial area of normal ovaries in H&E stained histological sections (Fig. B.5). The interstitial cells containing the foamy material in H&E stained sections are varied in shape and size (5-20 µm in diameter). The cells appear to be macrophages, inactive interstitial cells (cells waiting to be recruited as luteal cells), or fibroblasts.

F4/80 immunostaining shows that some, but not all, of the foamy cells appearing in H&E stained sections are macrophages (Fig. B.6). A large number of macrophages are seen in the interstitial tissue of normal ovary, corresponding to areas of bright fluorescence in TPEF images, and small foamy cells in H&E stained sections. The majority of the large foamy cells appearing in H&E stained sections of normal and carcinoma specimens do not stain positive as macrophages, and appear to be, instead, other ovarian interstitial cells.

Sudan Black B stained sections show positive lipid staining in small granules throughout the tissue, as well as positive lipid staining in the entire cytoplasm of some cells (Fig. B.7, B). The small granules seem to be the same size and distribution as the punctate fluorescence seen in TPEF images. The lipid-positive cells appear to be the same cells containing the foamy material in H&E stained sections. Further, staining with Sudan
Black B provides great contrast, revealing lipid positive foamy cells in normal ovary that were difficult to see in H&E stained sections.

To further elucidate the content of the lipid material as lipofuscin, carbol fuchsin staining was performed. Carbol fuchsin stained sections revealed staining patterns consistent with Sudan Black staining. Positive staining for lipofuscin was located not only in small granules, but also throughout the cytoplasm in clusters of ovarian interstitial cells (Fig. B.7, C).

Fluorescence images of unstained normal ovary from the fluorescence microscope show dim cellular fluorescence from all cells, small punctate fluorescence throughout interstitial tissue and bright cellular fluorescence from some clusters of interstitial cells (Fig. B.8, A). After staining with Sudan Black B, dim cellular fluorescence remains and all punctate and bright cellular fluorescence is blocked (Fig. B.8, C). This fluorescence imaging confirms that the foamy cells containing the fluorescent lipid material that stained with Sudan Black B are co-located with the bright fluorescent granules and foamy cells. In another section of normal ovary, fluorescence microscopy also shows small bright punctate fluorescence throughout tissue and clusters of brightly fluorescing cells (Fig. B.9, A). Similar to results from staining with Sudan Black B, after staining with carbol fuchsin the bright fluorescence is blocked, providing additional confirmation that the bright fluorescence is co-located with lipofuscin (Fig. B.9, C).

Changes in TPEF with age
Visual inspection of TPEF images of ovaries at three different ages (11, 13, and 15 months), did not reveal observable difference in the size, shape, or quantity of fluorescent regions associated with intracellular NADH, collagen, or blood vessel elastin. Visual examination of H&E stained histological sections also failed to reveal any observable differences in these tissue constituents. However, bright fluorescence associated with lipofuscin appeared to increase with age. With an increase in age, TPEF images of normal ovaries contained more small bright punctate fluorescent regions, as well as showed an increase in size of the large bright fluorescent regions (Fig. B.10).

Quantitative analysis of the TPEF images confirms that there is an increase in the number and size of brightly fluorescing regions with age. From 11 months to 13 months of age there was a statistically significant increase in all three parameters tested: the number of regions less than or equal to 36-pixel-area (p<0.0001), the number of regions greater than 36-pixel-area (p=0.0319) and the size of regions greater than 36-pixel-area (p=0.0123) (n[11 months]=70, n[13 months]=130). There was not a statistically significant change in any parameters from 13 to 15 months of age (n[13 months]=130, n[15 months]=69, p>0.05) (Fig. B.11).

Similar to findings from visual examination of TPEF images, visual examination of Sudan Black and carbol fuchsin stained sections of normal ovary reveal larger number and size of positively stained regions, indicating an increase in lipopigment accumulation with age (Fig. B.12). At 11 months of age, the lipofuscin-filled cells are very small in size and number and there is some small punctate staining. In addition, at 11 months of age,
the few lipofuscin-filled cells appear in small clusters. At 13 months of age, the cells containing lipofuscin appear larger in size and number than at 11 months. Further, the lipofuscin-filled cells are more widespread throughout the tissue. At 15 months of age, there are again more abundant and larger-sized lipofuscin-containing cells throughout the ovarian interstitial tissue.

Changes in TPEF with disease

Changes in TPEF as a function of disease were primarily associated with observed changes in morphology. Most of the tumors contained fewer or no luteal or granulosa cells, reducing the contribution of fluorescence associated with NADH. In addition, carcinoma specimens frequently had abundance of adipose cells and bands of connective tissue throughout the tumor, changing the morphology of NADH fluorescence and increasing the contribution of fluorescence associated with collagen. Furthermore, in tumors, an abundance of bright fluorescence from lipofuscin frequently masked NADH fluorescence.

Visual inspection of TPEF images revealed an apparent increase in bright fluorescence in nearly all disease types. DMBA effect, tubular adenoma, tubular adenoma with dysplasia and carcinoma appeared to have larger size and quantity of bright fluorescent regions (Fig. B.13). TA seemed to have the largest quantity of bright regions, with the regions dominating the entire field of view in some images. The sex-cord tumors appeared to contain the fewest bright regions of all tumors, with quantity of bright regions similar to normal tissue. TPEF images from each of the carcinoma specimens showed large
variability in fluorescence morphology. Two carcinoma specimens appeared similar, with large clusters of bright regions. The other carcinoma specimens had fewer bright regions and included fluorescence from bands of collagen and NADH in adipose cells, as confirmed by histology.

Quantitative analysis of TPEF images, all ages combined for each diagnosis (n given in Table B.1), confirmed a trend of larger and more numerous bright regions associated with disease (Fig. B.14). The average number of bright regions less than or equal to 36-pixel-area was greater than normal for DMBA effect, and lower than normal for tubular adenoma with dysplasia, sex-cord tumor and carcinoma, but the differences from normal were not significant (p>0.05). The average number of bright regions greater than 36-pixel-area was greater than normal for DMBA effect, tubular adenoma, tubular adenoma with dysplasia and sex cord tumor, and less than normal for carcinoma, but again the differences from normal were not significant (p>0.05). The average area of bright regions greater than 36-pixel-area was greater in all diseased tissue types as compared to normal, and this increase was statistically significant for tubular adenoma (p=0.0002).

Carbol fuchsin staining reveals lipofuscin morphology similar to the morphology of bright fluorescent regions seen in TPEF images. Further, examination of carbol fuchsin stained tissue sections supports the trend of increased lipofuscin accumulation with disease seen in the TPEF images (Fig. B.15). DMBA effect, tubular adenoma, tubular adenoma with dysplasia and carcinoma all appeared to have larger lipofuscin containing cells than normal tissue. Furthermore, many of the cells appear to have much darker and
denser staining than normal. The sex cord gonadal stromal tumors show few lipofuscin stained cells, in quantities similar to or less than normal. All diagnoses showed abundance of punctate lipofuscin staining throughout the tissue in varying quantities.

Discussion

In this study, we have shown the origin of TPEF signal in mouse ovaries at various ages and disease states. With TPEF excitation of 780 nm and collection of 405-505 nm, vitamins, amino acids and FAD have negligible signal [25, 28, 29] as compared to NADH, collagen and lipopigments. With an increase in age, normal ovaries show an increase in TPEF signal from lipofuscin and no changes in TPEF from NADH or collagen. Occurrence of disease caused a change in the fluorescence morphology. Many tumors had an increase in TPEF signal from lipofuscin as compared to normal ovary. Carcinoma specimens also showed an increase in TPEF signal from collagen. The low number of macrophages present in F4/80 staining of carcinoma, as compared to the number of cells staining positive for lipofuscin, indicated that in carcinoma specimens the majority of lipofuscin is not accumulated in macrophages, but rather in other native ovarian interstitial cells such as fibroblasts.

The average size and number of fluorescent cells and punctate material found by TPEF region analysis matched well with the morphology of lipofuscin seen by carbol fuchsin staining. However, there were large deviations from the averages in each group. This is due to the high variability in the location of the lipofuscin granules and foamy cells throughout the tissue. As seen by carbol fuchsin staining, some areas of tissue in a section
have areas with abundant punctate staining and many clusters of lipofuscin-filled cells as well as areas with less punctate staining and few or no lipofuscin-filled cells. Since the TPEF signal was collected from a small field of view (400 x 400 um), the number and size of fluorescent cells and punctate fluorescence was highly variable based on the location that was imaged. For example, only two carcinoma specimens showed an area with foamy cells in the TPEF images even though all of the carcinoma specimens had foamy cells visible in H&E and carbol fuchsin stained sections. Furthermore, all images vary in the amount of field of view containing signal and the number of fluorescent regions is highly dependent on this amount. The curved or varied topography of the tissue surface combined with the water immersion objective led to the large number of images without signal in the full field of view. To reduce the effects from within-tissue variability, multiple locations on the tissue must be imaged in order to get a complete understanding of the fluorophore content and morphology in the tissue. To obtain images with signal in the entire field of view a multifocal approach could be implemented, or a contact probe could be utilized to flatten the tissue so that the depth of imaging in the tissue is equal across the field of view. Another limitation of this study was the low number of adenocarcinomas that developed. More conclusive findings could be drawn from a larger sample size.

Previous literature has shown the presence of punctate fluorescence in human ovaries [13]. Large regions of bright fluorescence, as was seen in this mouse model, have not been described in human ovaries. However, accumulation of lipopigments has previously been found in genital system granulomas [22]. Furthermore, the trend of more lipofuscin
containing cells with age and disease follows the trend of lipofuscin accumulation with age and disease seen in the liver [28]. The significant increase in the size of foamy, lipofuscin containing cells with the development of a variety of tumors is likely due to heightened oxidative damage as a result of cell proliferation and death. This is supported by the accumulation of a large number of foamy cells in tubular adenoma, a quick-developing tumor, and carcinoma, an aggressive tumor, but less of such cells in the slow growing sex-cord tumors, which are generally benign.

TPEF images from normal mouse ovaries in this study look similar to TPEF images of normal human ovaries seen in previous literature, featuring dim cellular fluorescence from NADH and bright punctate fluorescence throughout the interstitial tissue [13]. In addition to normal human ovaries, Kirkpatrick et al. imaged human ovarian carcinoma, but they were interested in NADH fluorescence and processed the images to remove bright punctate fluorescence in carcinoma images prior to publication. Therefore, it is unknown if patterns of bright fluorescence, similar to the patterns seen in our mouse model, occur in human ovarian cancer.

The similarity in morphology of TPEF of the normal mouse and human ovary suggest that discoveries in this animal model could translate to humans. The usefulness of the model is further supported by the trend of increasing lipofuscin with age and disease, seen in humans [22, 30]. Furthermore, the rapid accumulation of lipofuscin with tumor development in this model suggests that lipofuscin content has potential as a diagnostic factor and may be a good early indicator of ovarian cancer in young patients, where age
is less likely to confound findings. In such patients, single or two-photon fluorescence imaging or spectroscopy could be implemented in vivo using minimally invasive techniques to detect lipofuscin accumulation. Animals with a high metabolic rate accumulate lipofuscin faster than animals with slower metabolic rate, so the accumulation of lipofuscin seen in these tumors is likely accelerated as compared to what may be seen in humans [31]. Additional studies are required to determine if lipofuscin accumulation commonly occurs with disease in human ovary. In the future, we would like to perform two-photon fluorescence imaging in human tissue to determine if lipofuscin may be a good diagnostic indicator of disease in human ovary.

References


Fig. B.1. (A) TPEF image showing dim fluorescence from a corpus luteum and granulosa cells surrounding a follicle in normal ovary, (B) H&E of normal ovary showing a corpus luteum and a follicle surrounded by granulosa cells. F=follicle, CL=corpus luteum. Scale bar equals 100 µm.

Fig. B.2. (A) TPEF image showing dim fluorescence from adipose cells and carcinoma cells, (B) H&E of carcinoma specimen with adipose cells and carcinoma cells. a=adipose cells, c=carcinoma cells. Scale bar equals 100 µm.
Fig. B.3. (A) TPEF image showing dim linear bands of fluorescence in a carcinoma specimen, (B) H&E showing collagen abundance in a carcinoma specimen. Arrow=collagen. Scale bar equals 100 µm.

Fig. B.4. (A) TPEF image showing collagen & elastin fluorescence from a blood vessel in a normal ovary. (B) H&E showing blood vessels in normal ovary. Arrow=blood vessel. Scale bar equals 100 µm.
Fig. B.5. (A) TPEF image showing bright punctate and bright cellular fluorescence in normal ovary, (B) H&E showing foamy cells in normal ovary. P=punctate fluorescence, Arrow=foamy cells. Scale bar equals 100 µm.
Fig. B.6. F4/80 positive macrophages stained brown in (A) normal ovary and (B) carcinoma. Corresponding H&E sections for (C) normal ovary and (D) carcinoma. Sections also contained foamy cells that are not macrophages. Arrowheads=macrophages, Arrow=foamy cells that are not macrophages. Scale bar equals 100 µm.
Fig. B.7. Normal ovary (A) Foamy appearing cells are present in H&E stained section (B) Lipid-filled cells and granules appear black in Sudan Black B stained section (C) lipofuscin appears purple in carbol fuchsin stained section. Squares show a zoomed in area to emphasize areas with many lipid-filled cells. Arrows point to granules. Scale bar equals 100 µm.

Fig. B.8. Fluorescence imaging shows co-localization of fluorescence and Sudan Black B stained lipid in normal ovary. (A) unstained ovary section under green light, (B) ovary section stained with Sudan Black B (bright field image), (C) Sudan Black B stained ovary section under green light. Scale bar equals 100 µm.
Fig. B.9. Fluorescence imaging shows co-localization of fluorescence and carbol fuchsins stained lipofuscin in normal ovary. (A) unstained ovary section under green light, (B) ovary section stained with carbol fuchsin (bright field image), (C) Carbol fuchsin stained ovary section under green light. Scale bar equals 100 µm.

Fig. B.10. TPEF images of normal ovary showing increase in lipofuscin fluorescence with age. (A) 11 months old, (B) 13 months old, (C) 15 months old. Scale bar equals 100 µm.
Fig. B.11. TPEF analysis results for normal ovary at each age. Bars indicate standard error of the mean. *Indicates significant difference from previous age (p<0.05).

Fig. B.12. Lipofuscin (in red/purple) accumulation increases with age. Normal ovary at (A) 11 months, (B) 13 months (C) 15 months of age. Scale bar equals 100 µm.
Fig. B.13. Representative aged-matched TPEF images for each diagnosis. (A) Normal (B) DMBA effect, (C) tubular adenoma, (D) tubular adenoma with dysplasia, (E) sex-cord tumor, (F) carcinoma. Scale bar equals 100 µm.
Fig. B.14. TPEF particle analysis results for each diagnosis. Bars indicate standard error of the mean. *Indicates significant difference from normal (p=0.0002).
Fig. B.15. Lipofuscin (stained red/purple) accumulates in cells and granules throughout the tissue of each diagnosis. (A) Normal (B) DMBA effect, (C) tubular adenoma, (D) tubular adenoma with dysplasia, (E) sex-cord tumor, (F) carcinoma. Scale bar equals 100 µm.
Table B.1. Number of ovaries and images with each diagnosis at each age. Number of images shown in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>DMBA Effect</th>
<th>Tubular Adenoma</th>
<th>Tubular Adenoma w/Dysplasia</th>
<th>Sex-cord Tumor</th>
<th>Carcinoma</th>
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<td>4 (30)</td>
<td>4 (20)</td>
<td>2 (42)</td>
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<tr>
<td>15 months</td>
<td>6 (69)</td>
<td>3 (30)</td>
<td>3 (21)</td>
<td>4 (28)</td>
<td>0 (0)</td>
<td>1 (14)</td>
</tr>
<tr>
<td>All Ages Combined</td>
<td>22 (269)</td>
<td>11 (95)</td>
<td>10 (63)</td>
<td>8 (48)</td>
<td>2 (42)</td>
<td>4 (57)</td>
</tr>
</tbody>
</table>
B.2 File Locations

Files associated with TPEF paper:
\MSPACMAN\Data\DroboData\JenWatson\Authored Papers\TPEF_exvivo

Subset of TPEF images stacks used for analysis (have had images with poor signal or artifact removed) are labeled by mouse ID# and R or L and are located in:
\MSPACMAN\Data\DroboData\Mouse_Study_2010Exvivo\Image_analysis\BrewDiagAnaly\brewdiagNA

Histology and special stain images are located in: \MSPACMAN\Data\(E)\DroboData\Mouse_Study_2010Exvivo\Histology and in: \MSPACMAN\Data\(E)\DroboData\JenWatson\Histology\n
ImageJ data (used for paper) and Matlab data (not used for paper) located in:
\MSPACMAN\Data\(E)\DroboData\Mouse_Study_2010Exvivo\Image_analysis\BrewDiagAnaly\brewdiagNA
B.3 Standard Operating Procedure for Sudan Black B stain for staining Lipids

SOP # B14-002

Materials:

1. Xylenes
2. Alcohols (70%, 80%, 90%, 95%, 100%, 100% EtOH)
3. Sudan Black B (Sigma Aldrich, St. Louis, MO) saturated (until precipitates) in 70% ethanol and filtered [stable for 6 months]
4. 70% ethanol
5. Tap water
6. 0.1N acetic acid: 2 ml glacial acetic acid 346 ml dH2O
7. 0.1N sodium acetate: 4.1 g NaAc to 500 ml dH2O
8. 0.1N acetate buffer: 148 ml 0.1N acetic acid to 352 ml sodium acetate (adjust to pH 5)
9. Methyl green
10. 90% acetone
11. 100% acetone
12. Permount

Procedure:

1. Dewax sections in xylene (10 min)
2. Rinse in alcohols (100%, 100%, 95%, 90%, 80%, 70% EtOH)
3. Place slides in humid chamber

4. Drop Sudan Black B stain directly on tissue using pipette (use wax pen as needed to prevent spillage)

5. Cover humid chamber and let slides stain with Sudan Black B overnight at room temperature

6. Differentiate in 70% ethanol until background appears pale gray

7. Wash in tap water

8. Acetate buffer for 5 minutes

9. 10 seconds in methyl green

10. 90% acetone for 10 seconds

11. 100% acetone for 10 seconds

12. Clear in xylene (10 minutes)

13. Mount using Permount and coverslips

Result:

Lipids & RBCs=Black

Background=green
B.4 Standard operating procedure for Ziehl-Neelsen modified to stain lipofuscin

(adapted from Pearse 1960)

SOP # B14-003

Materials:

1. Xylenes
2. Alcohols (70%, 80%, 90%, 95%, 100%, 100% EtOH)
3. Carbol fuchsin (Fluka Analytical, Sigma Aldrich, St. Louis, MO, #21820-100ML)
4. Tap water
5. Acid alcohol (1%HCL in 70% EtOH)
6. 0.25% methylene blue in 1% acetic acid
7. Permount & coverslips

Procedure:

1. Dewax sections in xylene (10 min)
2. Rinse in alcohols (100%, 100%, 95%, 90%, 80%, 70% EtOH)
3. Rinse in tap water
4. Place slides in humid chamber
5. Drop carbol fuchsin stain directly on tissue using pipette (use wax pen as needed to prevent spillage)
6. Cover humid chamber and let slides stain with carbol fuchsin overnight at room temperature
7. Wash well in tap water (until water runs clear)

8. Differentiate in acid alcohol until excess background staining is removed (1-3 minutes)

9. Wash in tap water

10. Counterstain with 0.25% methylene blue in 1% acetic acid (1 minute)

11. Wash in tap water

12. Quick wash in alcohols (three quick dips) (70%, 80%, 90%, 95%, 100%, 100% EtOH)

13. Clear in xylene (10 minutes)

14. Mount using Permount and coverslips

Result:

Lipofuscin=Deep red

Nuclei=blue

Background= pale pink to pale blue

RBCs=pale pink
B.5 Standard operating procedure for IHC staining for F4/80 macrophages

Using paraffin embedded tissues.

Antigen retrieval solutions and reagents:

**Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0):**

Tri-sodium citrate (dihydrate) --------- 2.94 g

Distilled water ------------------------ 1000 ml Mix to dissolve. Adjust pH to 6.0 with 1N HCl and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4 C for longer storage.

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td>xylene</td>
</tr>
<tr>
<td>10 min</td>
<td>xylene</td>
</tr>
<tr>
<td>5 min</td>
<td>100% EtOH</td>
</tr>
<tr>
<td>5 min</td>
<td>100% EtOH</td>
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<tr>
<td>5 min</td>
<td>99% EtOH</td>
</tr>
<tr>
<td>5 min</td>
<td>95% EtOH</td>
</tr>
<tr>
<td>5 min</td>
<td>90% EtOH</td>
</tr>
<tr>
<td>5 min</td>
<td>80% EtOH</td>
</tr>
</tbody>
</table>
5 min 70% EtOH

5 min ddH2O

3 x 3 min PBS wash

15 min 0.3% H2O2 in methanol - 500ul (30% H2O2)/50ml

3 x 3 min PBS wash

Antigen retrieval: Pre-heat 2 L beaker with Sodium Citrate Buffer until temperature reaches 95-100 °C.

Immerse slides in the beaker. Place the lid or foil loosely on the staining dish and incubate for 10-20 minutes.

Turn off water bath, remove beaker to room temperature, and allow the slides to cool for 20 minutes.

Rinse slides in water.

15 min RT 10% goat serum

3x3 min PBS wash

O/N, 4°C 1° antibody Antibody Type - _rat anti-mouse-F4/80 (Abd serotec, MCA497GA) Dilution: 1: 20: in 5%goat serum in DCF PBS

3 min X 3 DCF-PBS pH 7.4
30-60 min  RT  2° antibody Antibody Type - goat anti-rat IgG biotinylated (Vector Labs, BA-9401) in 2% BSA(with 10% goat serum)  1:500

5 min X 3  DCF-PBS pH 7.4

10 min  RT  Streptavidin

5 min X 3  DCF-PBS pH 7.4

1 - 30 min.  Substrate – DAB when light brown color is observed put in water.

(Dako )

5 min X 3  ddH₂O

1 min  hematoxylin

5min  running ddH₂O

Rinse  PBS

Dehydrate (70, 80, 90 100 5m in ea, xylene 5 min)

10 min  Xylene

Permoun & coverslip
APPENDIX C: *IN VIVO* TIME-SERIAL MULTI-MODALITY OPTICAL IMAGING IN A MOUSE MODEL OF OVARIAN TUMORIGENESIS

C.1 Manuscript: *In vivo* time-serial multi-modality optical imaging in a mouse model of ovarian tumorigenesis

The following manuscript has been submitted to the Journal of Biomedical Optics.
Title: *In vivo* time-serial multi-modality optical imaging in a mouse model of ovarian tumorigenesis

Authors: Jennifer M Watson, Samuel L Marion, Photini F Rice, David L Bentley, David G Besselsen, Urs Utzinger, Patricia B Hoyer, Jennifer K Barton

Abstract

Identification of the early microscopic changes associated with ovarian cancer may lead to development of a diagnostic test for high-risk women. Methods: In this study we use optical coherence tomography (OCT) and multiphoton microscopy (MPM) (collecting both two photon excited fluorescence [TPEF] and second harmonic generation [SHG]) to image mouse ovaries *in vivo* at multiple time points. We demonstrate the feasibility of imaging mouse ovaries *in vivo* during a long-term survival study and identify microscopic changes associated with early tumor development. These changes include alterations in tissue microstructure, as seen by OCT, alterations in cellular fluorescence and morphology, as seen by TPEF, and remodeling of collagen structure, as seen by SHG. These results suggest that a combined OCT-MPM system may be useful for early detection of ovarian cancer.

Keywords: optical coherence tomography, multiphoton, non-linear microscopy, two-photon, autofluorescence, cancer, optical biopsy

Introduction
There are more than 200,000 new cases of ovarian cancer and 100,000 deaths due to ovarian cancer each year worldwide.\textsuperscript{1} The high mortality rate is the result of frequent late diagnosis due to nonspecific symptoms and poor understanding of disease development and progression. Our goal is to use optical imaging to detect early cancerous changes in the ovary. By determining the microscopic changes that occur in early ovarian cancer, we hope to better understand disease progression and develop a minimally invasive diagnostic test for high-risk patients.

Due to the lack of availability of early carcinoma specimens in humans, we study ovarian tumor development using a mouse model. This model was developed through dosing with 4-vinylcyclohexene diepoxide (VCD) and 7, 12-dimethylbenz[a]anthracene (DMBA) and has been shown to lead to multiple tumor types in mice.\textsuperscript{2} The chemical VCD causes follicular degeneration, creating a quiescent state in the ovary, similar to menopause in humans. The chemical DMBA is a known carcinogen. Understanding changes in the post-menopausal-like ovary is useful in translating findings to women at risk for ovarian cancer, the majority of whom are peri- or post-menopausal.

Many optical imaging modalities including optical coherence tomography (OCT), confocal microscopy, fluorescence microscopy, and multiphoton microscopy (MPM), have shown promise for ovarian cancer detection.\textsuperscript{2-15} In this study, we use both OCT and MPM. OCT collects back-scattered light from index of refraction mismatches in the tissue and can achieve 2 mm imaging depth with resolution on the order of 10 µm. OCT has shown promise for cancer imaging\textsuperscript{16,17} and has been used by our laboratory and
others’ to study ovarian cancer both in animal models and in humans. In a rat model of ovarian cancer, OCT was able to identify image features that differed among normal, cystic sex cord-stromal tumors and solid sex cord-stromal tumors. In an in vivo human imaging study, Hariri et al. showed that OCT could distinguish normal from abnormal features in human ovary.

MPM is a non-linear imaging technique that uses infrared light to allow collection of images up to a hundreds of micrometers in depth with submicron resolution. MPM has been used for in vivo imaging of many tissues, including skin, kidney, brain, and ovary. Williams et al. found that MPM was useful for visualizing differences in normal ovarian surface epithelium and ovarian cancer in ex vivo human ovary and in vivo mouse ovary. Using MPM, Kirkpatrick et al. identified differences in the collagen structure, cellular organization and redox ratio of normal ovary and cancerous ovary biopsies from women. Imaging with OCT and MPM provides information on two scales and allows us to evaluate gross structural changes (via OCT) as well as microscopic changes in fluorophore concentration (via the MPM technique two-photon excited fluorescence [TPEF]) and collagen organization (via the MPM technique second harmonic generation [SHG]) in vivo without damage to the tissue.

Others have shown that single time-point (non-survival) imaging in vivo with OCT or MPM is possible in the mouse model, and imaging multiple times over weeks (in the brain) in mice is possible (through a cranial window). However, to our knowledge, no one has imaged ovaries at multiple time points in vivo long-term (over months). We show
the feasibility of imaging \textit{in vivo} with OCT and MPM at multiple time points in a mouse model that can develop ovarian cancer. Further, we find evidence of microscopic changes over time associated with disease development in mouse ovarian tissue.

Methods

\textit{Animals}

Protocols were approved by the University of Arizona Institutional Animal Care and Use Committee and experiments were performed per NIH guidelines. Forty-eight female B6C3F1 mice (age 28 days, Harlan, \textit{Dublin, VA, USA}) were housed in microisolators and allowed a seven-day acclimation period before initiating the experiment. The 35-day-old mice received intraperitoneal (IP) injections of VCD (Sigma Chemical Company, St. Louis, MO, USA), 160 mg/kg/day in sesame oil, or received sesame oil vehicle only as control, daily for 25 days. Four months after the end of IP dosing, animals received a single injection of DMBA (Sigma Chemical Company, St. Louis, MO, USA), 100 µg in 10 µl sesame oil, or 10 µl sesame oil vehicle only for controls, under the bursa of the right ovary. The left ovary was not injected or imaged \textit{in vivo} at any time; thus, left ovaries will not be discussed. There were four experimental groups: VCD and DMBA exposed (VCD/DMBA), VCD and vehicle injection (VCD/CON), DMBA and vehicle injection (CON/DMBA), or vehicle injection only (CON/CON), with 12 mice in each group. Mouse right ovaries were imaged \textit{in vivo} immediately before sub-bursal injection with DMBA, and at 5 and 7 months following DMBA injection (age 6, 11, and 13 months, respectively). Animals were euthanized after the final imaging time point at 13
months of age and ovaries were harvested immediately. Ovaries were also imaged *ex vivo* after excision.

*Surgical Access to Ovary*

Ovaries were accessed through a sterile surgical method for DMBA injection and imaging. Prior to surgical preparation mice were given 2% Avertin anesthetic (25 gm tribromoethanol in 15.5 ml 2-butanol; 2 ml stock in 100 ml sterile saline, Aldrich Chemical Company, St. Louis, MO, USA) .015 ml/gram body weight through IP injection. Surgical preparation of skin included shaving the right dorsal side of the body from the ribs to the hind limb with clippers and treating skin with Nair (Church & Dwight Co., Inc., Princeton, NJ, USA) hair remover for one minute to remove remaining hair. Nair was removed using water and cotton pads. Exposed skin was then disinfected by three repeats of swabbing with iodine followed by spraying with 70% ethanol.

Following surgical preparation, mice were transferred from the preparation area to the clean surgical area, placed on a custom-built heating pad and were given isoflurane, USP (JD Medical Dist. Co., Inc., Phoenix, AZ, USA), anesthetic through a nose cone and draped with a sterile surgical drape. Isoflurane was initially set at 2% volume (98% oxygen) to ensure surgical level of anesthesia (as confirmed by toe pinch) prior to skin incision. Following skin incision, isoflurane level was lowered to 1.5% volume (98.5% oxygen) for surgical maintenance. Anesthesia levels were monitored during surgery and imaging procedure by observing the animal’s breathing pattern and coloring.
For ovary access, a small incision was made in the skin between the caudal rib and hind-limb. Blunt dissection was used to release the skin from the peritoneal wall. Then a second incision was made in the peritoneum above the ovary. The ovary, which remained attached to the fallopian tube/uterine horn, was gently brought out of the peritoneal cavity and to the surface of the skin.

_Ovary isolation_

Following ovary retrieval, the ovary was rinsed with sterile saline and isolated from the body to reduce motion artifacts during imaging. For isolation the ovary was secured between a sterile coverslip and sterile spoon using gentle compression with sterile wire or rubber bands. The spoon spanned from the incision (where the ovary was held) to a post on the heating pad (where the spoon was held with a clamp).

Following isolation of the ovary, the animal was transported from the surgical suite to the microscope stage in the next-door room by securing the heating pad on a tray on top of the rolling anesthesia machine. Thus, the surgical level of anesthesia and body temperature were maintained during transport. Sterile bacteriostatic surgical lubricant (Surgilube, Fougera, Melville, NY, USA) was used on top of the coverslip for index matching with the water immersion multiphoton objective.

_Imaging_

The OCT and MPM instruments were arranged to share a microscope stage, with a rail system designed such that the animal (on the custom heating/anesthesia stage) could be
repeatedly translated from one imaging system to the other with the centers of their fields of view remaining co-localized. This arrangement allowed comparison of microscopic information obtained with the MPM to gross information obtained with the OCT, even though the field of view of the OCT was an order of magnitude larger than the MPM. Prior to each imaging session an imaging test with a phantom was performed to assure co-localization of the OCT and MPM images.

OCT and MPM time serial image sequences shown for each diagnosis are from the same ovary. Three dimensional OCT image cubes were obtained from near the center of the ovary, and depending on the size of the ovary, may cover the entire ovary. Single image slices from this cube in the \emph{en face} (parallel to the tissue surface) or cross-sectional (perpendicular to the tissue surface) direction are shown. Images were cropped as needed to remove areas without signal, or which contained tissues other than ovary (e.g. fallopian tube or fat). Multiple \emph{en face} MPM (TPEF and SHG) images were obtained from the surface of the tissue until signal was lost. Images presented are maximum-intensity Z-projections. Z-projections were performed in ImageJ (NIH) generally using the entire image stack. However, images with severe non-repeating motion artifact or confounders such as fur were removed from the stack prior to performing the Z-projection. Image insets, used to show detail, were obtained from single images in the stack. Scale bars shown in one panel of each figure apply to all the panels in the figure.

\textit{Optical coherence tomography}
Three dimensional optical coherence tomography imaging was completed with a swept source OCT system (OCS1050SS, Thorlabs, Newton, New Jersey, USA). The system operates in non-contact mode with a central wavelength of 1040 nm and spectral bandwidth of 80 nm. The axial scan rate was 16 kHz and the power on the sample was measured as 1.5 mW. The OCT system has 11 um transverse resolution and 9 um axial resolution in tissue. Imaging volume was 3 mm x 3 mm x 2.18 mm deep and 1024 x 1024 x 512 pixels.

Multi-photon microscopy

MPM imaging was completed with a TrimScope (LaVision BioTec, Bielefeld, Germany). The laser source was a Chameleon Ultra 2 (Coherent Inc., California, USA) and excitation wavelength was tuned to 780 nm. The objective was an XLUMPlan Fl, 20X, 0.95 numerical aperture, water immersion lens (Olympus, Tokyo, Japan). The TrimScope was configured to record two-photon excited fluorescence and second harmonic generation image data simultaneously with non-descanning reverse detection Galium Arsenide sensors (H7422A-40, Hamamatsu, Hamamatsu City, Japan). TPEF was collected with a 460/80 nm bandpass filter (Chroma, Bellows Falls, VT, USA). SHG was collected using dichroic mirror Di01-R405-25x36 (Chroma) and a 377/50 bandpass filter (Semrock, Rochester, NY, USA). Power on the sample was adjusted at the beginning of each imaging session to equal 20mW. Images were taken at 5 um or 10 um increments from the surface of the tissue to 60-100um depth. Each image was 400um x 400um in
size and 1041x1041 pixels. Gray scale was adjusted for display purposes (to limit saturation and improve contrast between areas with and without signal).

**Histology and Pathological Evaluation**

After harvesting and *ex vivo* imaging, ovaries were fixed in Bouin’s solution for 2-4 hours, transferred to 70% ethanol, dehydrated, embedded in paraffin blocks, and sectioned at 5 μm thickness. Orientation was carefully maintained on filter paper so that the imaged region of the ovary was known and *en face* sections were taken for histology. Multiple sections were mounted on glass microscope slides and covered with a layer of paraffin for preservation until sections were stained. Every 20th section was stained with hematoxylin and eosin for standard structure-based pathology evaluation. All abnormal specimens were evaluated by a board-certified veterinary pathologist to establish morphologic diagnoses.

**Results**

**Animals**

Of the 48 animals in the study, a total of 44 right ovarian specimens were collected (all specimens discussed are from the right side of the animal). Four ovary specimens were not collected due to inability to locate the ovary.

The specimens were diagnosed per pathologic findings into the following categories: normal (cycling) (n=14), atrophic (acyclic) (n=6), atrophic with tubular hyperplasia
(n=15), cystic tumor (n=4), fibrosarcoma tumor (n=2), or granulosa cell tumor (n=3).

Table C.1 shows the respective dosing groups for each diagnosis.

Table C.1. Number of ovaries with each diagnosis in each dosing group.

<table>
<thead>
<tr>
<th>Dosing Group</th>
<th>Normal</th>
<th>Atrophic</th>
<th>Tubular Hyperplasia</th>
<th>Cystic Tumor</th>
<th>Fibrosarcoma Tumor</th>
<th>Granulosa Cell Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON/CON</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VCD/CON</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CON/DMBA</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>VCD/DMBA</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

H&E stained sections of each diagnosis are shown in figure C.1. Normal mouse ovary contained an epithelial layer (generally 1-2 cells deep) and a highly cellular stroma containing follicles and corpora lutea [Fig. C.1 A]. Ovaries diagnosed as atrophic had few-to-no developing follicles or corpora lutea and consisted primarily of ovarian interstitial tissue surrounded by a layer of germinal epithelial cells [Fig. C.1 B]. Small degenerate (atretic) follicles without oocytes were frequently visible throughout the atrophic tissue. Some atrophic ovaries had a proliferation of the epithelial layer. Atrophic ovaries also frequently contained lipofuscin-filled stromal cells and macrophages that appeared large golden-brown or light-pink colored and foamy in H&E stained sections.

Atrophy accompanied by tubular hyperplasia (referred to as tubular hyperplasia) was characterized by follicle and corpora lutea depletion (resulting from VCD exposure) and
multiple epithelial-cell-lined tubules traversing throughout the ovarian stroma, from the surface of the ovary to deep in the ovary [Fig. C.1 C]. Tubular hyperplasia can occur naturally in elderly mice, but in this study was likely caused (or accelerated) primarily by ovarian senescence resulting from exposure to the chemical VCD. Ovaries with cystic tumors contained fluid or blood filled cavities of variable size with smooth walls [Fig. C.1 D]. The cystic tumor shown had a larger number of variable sized cysts. Most of the large cysts contained follicular cells and blood and the small cysts contained follicular cells and fluid. Fibrosarcoma, a malignant tumor characterized by immature proliferating fibroblasts, appeared as dense spindle-shaped cells, sometimes in a whorling pattern [Fig. C.1 E]. The fibrosarcoma shown also contained multiple small cysts. Cells in granulosa cell tumors resembled the granulosa cells that nourish developing follicles and were organized in multiple follicle-like clusters [Fig. C.1 F]. The follicle-like cell clusters were of variable size and some, but not all, contained oocytes. Granulosa cell tumors contained round cells with centrally located nuclei and sometimes contained mitotic figures.
Fig. C.1. H&E stained sections for each diagnosis. Insets show higher magnification. A) Normal cycling (n=14), B) atrophic (n=6), C) tubular hyperplasia (n=15), D) cystic tumor (n=4), E) fibrosarcoma (n=2), F) granulosa cell tumor (n=3).
A total of 37 animals survived to the third imaging time point. Reasons for not surviving to the third time point were unexpected death or early sacrifice due to bleeding during surgery (n=7), or early sacrifice due to a large tumor found at the second imaging time point (n=4). All animals were imaged at one or more time points. Forty-seven ovaries were imaged at two or more time points with at least one imaging modality. Twenty-three ovarian specimens were imaged at two time points with both OCT and MPM (6 normal, 7 tubular hyperplasia, 3 cystic, 3 granulosa cell tumors, 3 atrophic, 1 fibrosarcoma). The reasons for obtaining only one or two time points from some mice included early sacrifice or inability to locate or secure the ovary for imaging at the second or third time point (due to adhesions). Nineteen ovaries were imaged at all three time points with at least one imaging modality. Only one imaging modality was used in some mice due to loss of ovary isolation or technical difficulties. Twelve ovaries were imaged at all three time points with both OCT and MPM. However, for three of these animals, inspection of the OCT images at the second time point revealed fat or fallopian tube rather than ovarian tissue in the center of the field of view, leaving nine image sequences of ovarian tissue at all three time points by both OCT and MPM (5 normal, 4 tubular hyperplasia).

*Image Analysis*

In this section, the features of a normal mouse ovary as seen in OCT and MPM images are described and time serial OCT and MPM images from all six diagnoses are presented in turn. Three time point images are shown for normal and tubular hyperplasia. Two time point images are shown for all other diagnoses owing to early sacrifice or difficulty
locating or securing the ovary for imaging. The last time point images shown were recorded *ex vivo*, except where *ex vivo* images were not available (granulosa cell tumor), in which case the *in vivo* image is shown. *Ex vivo* images have reduced motion artifact as compared to *in vivo* images.

*Normal Ovary Features*

A cross-sectional OCT image of a CON/CON normal ovary showed multiple fluid-filled follicles of various sizes and homogenous interstitial tissue [Fig. C.2 A]. An *en face* OCT image showed multiple follicles and corpora lutea (CL) of various sizes and stages of development, as well as connective tissue and fat [Fig. C.2 B]. The epithelial cell layer was not easily distinguished in OCT images, although the edges of the tissue in the *en face* image appeared to have slightly more intense scattering than the stroma. The stroma showed homogenous intensity with the exception of various-sized follicles and CL.

Fig. C.2. Normal ovary OCT images, A) cross-section, B) en face section. F=follicle, CL=corpus luteum, CT=connective tissue or fat, arrow points to bright edge.
A TPEF image of the surface of a normal ovary visualized epithelial cells, which appeared as a ring of intracellular fluorescence surrounding dark, round, centrally located nuclei [Fig. C.3 A]. TPEF images obtained from below the surface of the ovary revealed stromal cells, which also appeared as bright rings (usually oval-shaped) around dark, round, centrally located nuclei [Fig. C.3 B]. Some TPEF images of normal ovary showed large round cells of corpora lutea, which appeared slightly brighter than other cells. Additionally, some TPEF images revealed the variety of cells making up follicles, including the spindle and round-shaped theca cells, round-shaped follicular (granulosa) cells and the oocyte (not shown), which all appeared of similar brightness. TPEF images displayed extremely intense punctate or full-cell fluorescence from lipofuscins located in ovarian stromal cells. An SHG image of the surface of the ovary showed very dense collagen, especially at what appeared to be the epithelial-stromal boundary [Fig. C.3 C]. The collagen fibers at the surface were thin and linear and interweave with each other to form a tight-knit, net-like structure. The collagen in the stroma had a similar thin, linear (or slightly wavy) appearance but had gaps in the collagen where functional ovarian structures, such as follicles and corpora lutea, were located [Fig. C.3 D]. The gaps in the collagen were of various sizes depending on the sizes of the structures (could be up to 400 µm for largest follicles).
Fig. C.3. Normal ovary MPM images. A) TPEF from surface of the ovary. B) TPEF image from deeper in the ovary. C) SHG from the surface of the ovary. D) SHG from deeper in ovary. E=epithelial cells, CL=corpus luteum, F=follicle, S=stroma, wide arrow points to bright cells in stroma, chevron points to theca cells, thin arrow points to granulosa cells, arrowhead points to thin net-like collagen structure, five-point stars indicate gaps in collagen structure in the stroma.

Normal Ovary Time Series

Consecutive three time point images of a CON/CON ovary that was diagnosed as normal at the end of the study showed that normal ovaries maintain an organized structure at all
three imaging time points [Fig. C.4]. In the normal ovary shown, follicles and corpora lutea were visible in OCT images from all three time points. Further, follicles were present in multiple stages of development. In the first and second time point OCT images, fat and connective tissue were seen on one side of the ovary.

Fig. C.4. Normal ovary (CON/CON) time serial OCT images. A) 6 months, B) 11 months, and C) 13 months of age. F=follicle, CL=corpus luteum, CT=connective tissue or fat.

MPM images showed an organized microscopic structure at each imaging time point in the normal ovary [Fig C.5]. TPEF images showed abundant cellular and collagen fluorescence [Fig. C.5 A, B, C]. Further, epithelial and stromal cells were visible in TPEF images. The first time point TPEF image showed collagen fluorescence and dimly fluorescing epithelial cells near the surface of the ovary and both dimly fluorescing and brightly fluorescing cells in the ovarian stroma [Fig. C.5 A]. The first time point image was slightly blurred from motion artifact. At the second time point dim collagen fluorescence, dim cellular fluorescence and abundant bright cellular fluorescence were visible, although, the image displays some evident motion artifacts from breathing [Fig. C.5 B]. The third time point TPEF image appeared similar to the first time point with dim fluorescence from epithelial cells at the surface of the ovary and brighter fluorescence
from cells in the stroma [Fig. C.5 C]. Sharper cell boundaries were seen because there was no motion artifact in these ex vivo images. SHG images from all three time points showed thin, linear, tight-knit, net-like collagen structure present throughout the ovary [Fig. C.5 D, E, F]. The third time point SHG image showed thin linear fibers near the surface of the ovary and wavier fibers, surrounding multiple holes (corresponding to location of cells in TPEF image) in the deeper part of the ovary [Fig. C.5 F].
Fig. C.5. Normal ovary (CON/CON) time serial MPM maximum intensity projection images. A-C) TPEF images. D-F) SHG images. A &D from first time point, B & E from second time point and C & F from third time point. Insets show detail from a single slice in stack.

Atrophic Ovary

Two time point OCT images of an ovary, which belonged to the CON/DMBA group, that was diagnosed as atrophic at the end of the study showed a change in the gross structure of the ovary from the first to the third time point [Fig. C.6]. The first time point (before any dosing) OCT image showed a normal-looking ovary with multiple follicles [Fig. C.6 A]. The third time point image, taken 7 months after DMBA injection, showed the absence of follicles or corpora lutea, reduced ovary size (also visible by the eye) and somewhat inhomogeneous scattering intensity [Fig. C.6 B]. The third time point OCT image also revealed a few small degenerate (atretic) cysts and some fat and connective tissue attached to the ovary.
Fig. C.6. Atrophy (CON/DMBA) development in time serial OCT images. A) 6 months (before DMBA dosing) and at B) 13 months of age (after DMBA dosing). F=follicle, CL=corpus luteum, CT=connective tissue, arrows point to small cysts.

TPEF images of the CON/DMBA ovary at the first time point (before DMBA dosing) showed collagen fluorescence and some dim cellular fluorescence as well as bright punctate fluorescence in amounts similar to those seen in normal ovary at the same age [Fig. C.7 A]. The TPEF image from the third time point showed abundant, large, brightly fluorescing stromal cells that were not present at the first time point [Fig. C.7 B]. SHG images from the first and third time point showed thin, tight-knit, normal appearing collagen [Fig. C.7 C, D]. The third time point SHG also showed some areas of wavy collagen and small holes in the collagen structure that may correspond to small cysts [Fig. C.7 D]. The saturated spots seen in the first time point SHG were artifacts and fluorescence that overlapped the SHG collection wavelengths.
Fig. C.7. Atrophy (CON/DMBA) development in time serial MPM maximum intensity projection images. A-B) TPEF. C-D) SHG. A & C are from first time point (before DMBA dosing) and B & D are from third time point (after DMBA dosing). Insets show detail from a single slice in the stack.

*Tubular Hyperplasia*
Three time point OCT images of a VCD/CON ovary diagnosed as having tubular hyperplasia at the end of the study showed a change in ovarian structure as compared to normal ovary at all three time points [Fig. C.8]. In an OCT image from the first time point (four months after completion of VCD injections) the ovary did not contain follicles or corpora lutea and had inhomogeneous scattering intensity, giving it a rough appearance [Fig. C.8 A]. Additionally, the ovary had an irregular surface due to small invaginations, which was particularly noticeable in contrast to the smooth ovarian bursa (a protective layer present on mouse ovaries) covering the ovary. A few small cysts and one large cyst near the surface of the tissue were also visible. The second time point OCT image showed multiple small invaginations in the ovary under the ovarian bursa and possibly some small cysts throughout the ovary [Fig. C.8 B]. Further, the OCT image had inhomogeneous scattering intensity in the interior of the ovary resulting in a rough texture, as was seen at the first time point. At the third time point the OCT image revealed an inhomogeneous scattering intensity throughout the ovary with some small cysts, as were seen in previous time points, and an extremely irregular (tubular) surface with numerous large tubules invaginating deep into the ovary [Fig. C.8 C]. The invaginations seen with OCT were not visible by the eye at any time point.
Fig. C.8. Tubular hyperplasia (VCD/CON) development in time serial OCT images. A) 6 months, B) 11 months and C) 13 months of age. Cy=cyst, chevrons point to bursa, thin arrows point to small invaginations, thick arrows point to large invaginations.

A TPEF image of this ovary at the first time point (after VCD dosing) showed some collagen fluorescence, dim cellular fluorescence and bright cellular fluorescence [Fig. C.9 A]. The cells containing the bright fluorescence were of normal size but appeared more numerous than what was seen in normal ovary at the same time point. Although the cells appeared normal, some areas had tubules visible (Fig. C.9 A, A insets). The predominant signal in TPEF images at the second and third time point was the abundant bright cellular fluorescence, appearing in clusters of larger-than-normal cells [Fig. C.9 B, C]. Tubules were also visible in TPEF images at the second and third time point. SHG images showed a change in collagen over time. The SHG image at the first time-point showed collagen similar to normal with tightly packed, thin, linear fibers [Fig. C.9 D]. The second time point SHG image showed a more diffuse collagen structure where there appeared to be less collagen and fibers were spread farther apart, although fibers remained thin and linear [Fig. C.9 E]. At the third time-point, there was less collagen and the fibers had a tangled appearance with clumps of curved fibers spaced far apart [Fig. C.9 F].
Fig. C.9. Tubular hyperplasia (VCD/CON) development in time serial MPM maximum intensity projection images. A-C) TPEF. D-F) SHG. A & D are from the first time point. B & E are from the second time point and C & F are from the third time point. Insets show detail from a single slice in the stack. Arrows point to hypointense tubules.

_Cystic Tumor_

Two time point OCT images of a VCD/DMBA ovary diagnosed with a cystic tumor showed differences from normal ovary. The first time point (after VCD dosing) OCT
image showed ovarian tissue without follicles or corpora lutea [Fig. C.10 A]. These indications of ovarian atrophy, along with the inhomogeneous scattering throughout the tissue, made the OCT images appear similar to the first time point OCT images of ovarian tissue that developed tubular hyperplasia [Fig C.8 A]. However, the ovary that went on to develop a cystic tumor had more numerous small cysts visible at the first time point [Fig. C.10 A]. In addition to ovarian tissue, the first time point OCT image of the cystic ovary contained fat, connective tissue and fallopian tube. The second time point image (after DMBA dosing) showed hyper-intense scattering in some areas and many variable sized/shaped cystic cavities filled with cells and fluid [Fig. C.10 B]. The ovary also appeared much larger (by the eye) at the second time point than the first time point, thus the animal was euthanized at the second time point.

Fig. C.10. Cystic tumor (VCD/DMBA) development in time serial OCT images. A) 6 months, B) 11 months of age. CT=connective tissue, FT=fallopian tube, arrows point to small cysts.
A TPEF image from the first time point, of the VCD/DMBA-dosed ovary that developed a cystic tumor, showed collagen fluorescence, dim cellular fluorescence with dark nuclei in the cells and some areas of bright punctate fluorescence [Fig. C.11 A], consistent with features seen in other first time point images of ovaries dosed with VCD. At the second time point, TPEF showed two large follicle-like cysts, larger cells, and more abundant bright fluorescence [Fig. C.11 B]. SHG images from the first time point showed slightly wavy collagen and large vessels [Fig. C.11 C]. SHG from the second time point showed linear to wavy, tight-knit collagen, slightly thicker and more abundant than collagen seen in normal ovary [Fig. C.11 D].
Fig. C.11. Cystic tumor (VCD/DMBA) development in time serial MPM maximum intensity projection images. A-B) TPEF. C-D) SHG. A & C are from the first time point and B & D are from the second time point. Insets show detail from a single slice in the stack. Thin arrows point to blood vessels, wide arrow points to cells along the edge of a large follicle-like cyst.

*Fibrosarcoma Tumor*
Time serial images of a CON/DMBA-dosed ovary diagnosed with fibrosarcoma at the end of the study showed a change in ovarian structure from the first to the third time point [Fig. C.12]. At the first time point, OCT images showed a normal appearing ovary with follicles and corpora lutea [Fig. C.12 A], as would be expected since the animal has not yet been dosed with chemicals. The third time point OCT image (after DMBA dosing) showed homogenous tissue with hyper-intense scattering, some small cysts, an irregular edge and some deep invaginations [Fig. C.12 B].

The first time point TPEF image of the CON/DMBA ovary appeared normal and showed dimly fluorescing cells, some bright punctate fluorescence and some collagen.
fluorescence [Fig. C.13 A]. The first time point image also had a developing follicle visible. The third time point TPEF image (after DMBA dosing) showed dim cellular fluorescence with the cells having a different morphology (spindle shaped with larger nuclei) than normal and a few areas containing larger cells with bright fluorescence [Fig. C.13 B]. The first time point SHG image showed thin, linear collagen fibers that are tightly-packed and appear normal [Fig. C.13 C]. The third time point SHG showed linear to wavy collagen fibers that are more spread out and tangled than normal [Fig C.13 D].
Fig. C.13. Fibrosarcoma (CON/DMBA) time serial development in MPM maximum intensity projection images. A-B) TPEF. C-D) SHG. A & C are from the first time point and B & D are from the third time point. Insets show detail from a single slice in the stack. Thin arrows point to spindle-shaped cells.

Granulosa Cell Tumor
First and second time point OCT images of a VCD/DMBA ovary that developed a granulosa cell tumor showed a difference in structure from normal ovary [Fig. C.14]. The first time point OCT image (after VCD dosing) showed an atrophic appearing ovary, similar to other first time point OCT images of VCD-dosed ovaries, with smaller volume than normal ovary, no follicles or CL and some fat and connective tissue attached to the ovary [Fig. C.14 A]. The image also had inhomogeneous scattering intensity resulting in a rough texture. The second time point OCT image (after DMBA dosing) looked more homogenous with a characteristic sparkling texture owing to very intense punctate scatterers throughout the tissue [Fig C.14 B]. The ovary also appeared slightly larger than the previous time point (by the eye), thus the animal was euthanized at the second time point.

![OCT images of a VCD/DMBA ovary](image)

Fig. C.14. Granulosa cell tumor (VCD/DMBA) development in time serial OCT. A) 6 months, B) 11 months of age. Thin arrows point to punctate scatterers.
The first time point TPEF image of the VCD/DMBA ovary that developed a granulosa cell tumor showed collagen florescence, some bright punctate fluorescence and bright full-cell fluorescence [Fig. C.15 A]. The cells with bright full cell fluorescence were round to oval with prominent nuclei and were extremely large compared to normal cells. The second time point TPEF showed bright full-cell fluorescence [Fig. C.15 B]. Further, the cells maintained the large prominent nuclei and were organized in round clusters, characteristic of a granulosa-cell tumor. SHG images showed tightly packed linear collagen at the first time point that appeared slightly thicker than normal [Fig. C.15 C]. At the second time point there was less abundant collagen that appeared less tightly packed than at the previous time point [Fig. C.15 D].
Fig. C.15. Granulosa cell tumor (VCD/DMBA) development in time serial MPM maximum intensity projection images. A-B) TPEF. C-D) SHG. A & C are from the first time point and B & D are from the second time point. Insets show detail. Panel B inset shows a cluster of granulosa cells.

The characteristic features of ovarian tissue with each diagnosis are summarized in table C.2.
<table>
<thead>
<tr>
<th>DIAGNOSIS</th>
<th>OCT CHARACTERISTICS</th>
<th>TPEF CHARACTERISTICS</th>
<th>SHG CHARACTERISTICS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Ovary</td>
<td>Low intensity scattering, homogenous interstitial tissue, follicles, corpora lutea visible.</td>
<td>Dim cellular fluorescence with round centrally located nuclei (epithelial, follicular and stromal cells), bright fluorescence from CL cells, some stromal cells are slightly larger and have extremely bright fluorescence (punctate or entire cell filled).</td>
<td>Collagen appears as thin, linear or slightly wavy, with fibers packed into a tight, net-like structure near the surface and some gaps in the collagen structure in the stroma.</td>
</tr>
<tr>
<td>Atrophic Ovary</td>
<td>Areas of inhomogeneous scattering intensity, no follicles or CL, smooth surface.</td>
<td>Abundant large, extremely bright stromal cells.</td>
<td>Tightly packed, thin, curved or wavy collagen fibers.</td>
</tr>
<tr>
<td>Tubular Hyperplasia</td>
<td>Inhomogeneous scattering intensity. Rough surface with many small invaginations and some deep invaginations.</td>
<td>Tubules, dimly fluorescing stromal cells intermixed with clusters of large, extremely bright stromal cells.</td>
<td>Collagen is thin, linear or curved, with much less collagen present than normal. Fibers appear as tangled masses, spread far apart.</td>
</tr>
<tr>
<td>Cystic Tumor</td>
<td>Areas with hyperintense scattering, multiple variable sized solid and hollow (fluid filled) masses.</td>
<td>Many follicle-like structures and many stromal cells with extremely bright fluorescence.</td>
<td>Collagen appears slightly more abundant than normal. Fibers appear thin, linear or slightly wavy and are tightly packed in a net-like structure.</td>
</tr>
<tr>
<td>Fibrosarcoma Tumor</td>
<td>Homogenous tissue with hyperintense scattering, some small cysts and a very rough</td>
<td>Homogenous dim fluorescence, spindle shaped cells, large nuclei.</td>
<td>Spread out tangles of collagen fibers.</td>
</tr>
</tbody>
</table>
Granulosa-cell Tumor  | Bright punctate scatterers give a sparkling appearance to the tissue. | Round clusters of cells with large prominent nuclei. | Thin linear or wavy fibers, less tightly packed than normal.

Discussion

This study has demonstrated the feasibility of imaging mouse ovaries *in vivo* at multiple time points throughout an animal’s lifetime. Owing to the ability to image the same ovary at multiple time points, we were able to identify changes in ovarian microstructure with disease development. Further, multiple time point imaging allowed us to determine the time scale for microscopic changes associated with disease development. We have found that microscopic changes can be visualized with OCT and MPM before changes are seen by the eye. OCT provided cross-sectional micrometer-scale resolution of the entire mouse ovary but could not visualize cells. MPM enabled visualization of the epithelial surface and stromal cells up to 100 µm deep into the mouse ovary with sub-micrometer resolution. The pair of imaging modalities allowed for gross scanning of the entire ovary as well as zoomed-in microscopic information of endogenous fluorescence and collagen morphology.

*Normal*

All CON/CON ovaries were diagnosed as normal at the end of the study, as would be expected since spontaneous disease in this mouse strain is rare. The only change seen in
CON/CON ovaries over the duration of the study was an increase in bright cellular fluorescence from accumulation of lipofuscins [Fig 5 A, B, C], commonly seen with an increase in age, and noted earlier in our ex vivo studies.\textsuperscript{24} Four CON/DMBA ovaries were also diagnosed as normal at the end of the study. Although DMBA dosing usually caused follicular and corpora lutea degeneration, even after DMBA dosing, some ovaries contained a few remaining follicles or corpora lutea and were considered normal rather than atrophic. Images are not shown from DMBA-dosed ovaries diagnosed as normal; however image characteristics were similar to the CON/CON ovary shown in figures 4 and 5, but with fewer follicles and corpora lutea.

\textit{Atrophy}

Dosing with either VCD or DMBA caused a depletion of follicles and corpora lutea, a condition labeled as atrophy. By the first imaging time point (4 months after VCD dosing) VCD-dosed ovaries were expected to be follicle deplete, as a previous study has shown complete depletion of follicles by 58 days following VCD dosing.\textsuperscript{25} As expected, complete follicle depletion was seen at the first time point in VCD-dosed ovaries, as evidenced by lack of follicles or corpora lutea seen in OCT images [Figures 8 A, 10 A, 14 A]. One VCD-dosed ovary was diagnosed as atrophic (without other abnormalities present) at the end of the study. All other VCD-dosed ovaries developed tubular hyperplasia or other tumor types by the end of the study. Animals that were dosed with DMBA alone (after the first imaging time point) usually showed follicle depletion at the second or third time point. Thus, the difference between DMBA-dosed ovaries diagnosed
as normal and those diagnosed as atrophic is that atrophic ovaries had zero visible functional follicles (as evidenced by lack of oocytes in histology) or corpora lutea and frequently had proliferation of the germinal epithelial cell layer (seen in histology).

In both VCD and DMBA induced atrophy, OCT images revealed a smooth surface of the ovary and inhomogeneous scattering intensity resulting in a rough texture in the interior of the ovary. Further, starting at the first (in VCD-dosed) or second (in DMBA-dosed) imaging time point, TPEF images showed an increase in bright cellular fluorescence from lipoproteins. The increase in lipofuscins in atrophy was much more noticeable than the accumulation seen with age in the normal ovary. This finding was expected as an increase in endogenous fluorescence from accumulation of lipofuscins was seen in TPEF images of atrophied ovaries in a previous study. SHG images of VCD-dosed and DMBA-dosed ovaries that displayed atrophy at the first or subsequent time points have similar collagen to normal, displaying thin fibers. However, SHG images indicated some changes in collagen structure as evidenced by wavy fibers and a change in spacing between fibers as compared to normal in first and second time point images of VCD-dosed ovaries [Fig. 9 D, 11 C, 15 C] and second time point images of DMBA-dosed ovaries [Fig. 9 E].

Tubular Hyperplasia

VCD treatment most often led to the development of tubular hyperplasia by the final time point. This outcome is consistent with VCD dosing results seen in previous ex vivo studies at seven and nine months following VCD dosing. OCT and MPM provided evidence of very early tubule development, only 4 months after VCD dosing. The
increase in number and size of tubules was evident with each consecutive time point as seen by the increasing surface irregularity, deepening surface invaginations and inhomogeneous scattering in the stroma visible with OCT. Additionally, disease development was seen in TPEF images as an increasing bright cellular fluorescence from lipofuscins and SHG images as collagen transformed from thin, linear and tightly packed to tangled or diffuse with large gaps between clusters of fibers.

Other Tumors

The other tumors that developed were large enough to see an increase in size by the eye at the last imaging time point. However, images of each of these tumors showed evidence of tumor development before this increase in size, or provided evidence of tumor type before histology was available. In the ovary that developed a cystic tumor, the first time point OCT images showed many more small cysts than were seen in other VCD-dosed ovaries, indicating the initial development of the cystic tumor before the tumor was large enough to seen by the eye. Additionally, SHG showed wavier than normal collagen at the first time point. Early images of fibrosarcoma were not available. However, the last time point images showed characteristics specific to fibrosarcoma, such as the spindle-shaped cell morphology seen in TPEF images and the homogeneous scattering and irregular surface seen in OCT images. Images of the granulosa cell tumor showed early changes, evidenced by change in cell morphology seen in TPEF images at the first time point, as well as a possibly diagnostic sparkling appearance seen in OCT images at the second time point.
Study Limitations

This study suffered from three limitations that can be addressed in future studies. First, adhesions resulted in attachment of the ovary to the surrounding fat and other organs, leading to difficulty of obtaining in vivo images at all three time points. In future studies, we will utilize IP injected fluids or other materials indicated for preventing adhesions. Second, some images were of sub-optimal quality resulting from low signal and motion artifacts. Current studies suggest that the MPM power may be increased without affecting cell viability.\textsuperscript{26-29} Using larger average power would enable higher signal levels, faster scanning, elimination of line averaging and reduction in motion artifacts. The third limitation was the low incidence of adenocarcinoma in this mouse model.\textsuperscript{2} One animal model that would improve this study is the SV40 Tag MISIIR model developed by Connolly et al., which spontaneously develops bilateral ovarian adenocarcinoma.\textsuperscript{30}

In summary, we have successfully imaged mouse ovaries \textit{in vivo} at multiple time points using OCT and MPM. Additionally, we have identified microscopic changes from the first to second and third time points. Changes in the ovarian surface and the texture of the stroma were visible in OCT images at early stages of tumor development. Further, the majority of these microscopic surface changes were never visible by the eye. Alterations in ovarian microstructure, including cell and collagen morphology, were identifiable using MPM at early time points, before macroscopic changes were visible by the eye. These results suggest that visualizing microscopic changes associated with early disease development is possible and may be useful in humans. Previous studies have used
laparoscopic OCT and MPM endoscopes for \textit{in vivo} imaging.\textsuperscript{5, 31} However, MPM endoscopes would need to be made longer to reach the human ovary. Additionally, an OCT-MPM scope for human ovary imaging should include an articulating tip for imaging the fallopian tube, since there is evidence that cancer can originate not only from the ovarian surface epithelium, but also the fallopian tube.\textsuperscript{32, 33} Successful implementation of an endoscopic OCT-MPM for imaging the ovary and fallopian tube may make monitoring possible for women at high risk for ovarian cancer who wish to avoid or delay time to oophorectomy.

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References:


C.2 File Locations

Files associated with *in vivo* paper:

`
\MSPACMAN\Data(E)\DroboData\JenWatson\Authored Papers\in_vivo_ms`

Mouse study *in vivo* data including H&E stains, MPM data (raw data organized by acquisition date and copied into folders labeled with group [G4, S1, S2, S3] and time point [TP 1, 2, 3]) and OCT data (raw data organized by group and time point then by acquisition date) located in:

`
\MSPACMAN\Data(E)\DroboData\Mouse_Study_2010_In_vivo`

Files associated with dissertation are located in:

`
\MSPACMAN\Data(E)\DroboData\JenWatson\Dissertation`
C.3 Standard operating procedure for the multiphoton microscope

SOP# B02-008b

Supplies

1. Sample
2. Water
3. Lens paper
4. Isopropyl alcohol

Equipment

1. Multiphoton Microscope
2. 20x, 0.95 NA Olympus objective (water immersion)
3. Power meter

Procedures

Turn on and set-up the multiphoton microscope system

1. Ensure the chiller is turned on and reads 19 C.
2. Turn on the Laser warning sign (red switch near door).
3. Check that the slider to high sensitivity ports is closed.
5. Turn on the main power strip.
6. Turn the Laser key switch to “on.”
7. Open Inspector Pro software.
9. Under measurement mode, select 3D-scan.
10. In xy-scanner window, under SB scanmode tab, select size 400 x 400 um, pixel 1020 x 1021, frequency 800, line average 1.
11. In PMT window select HS_1 and HS2 and any other PMTs you are going to use and set PMT gains to 80.
12. In TriMScope window select open S&P, set beam diameter to pos5 and laser power to 1%.
13. In Laser window, set wavelength to 780 nm. Select system on and shutter open.

Setting laser power

1. Place power meter sensor under objective and bring objective close to (but not touching) sensor.
2. Turn on power meter
3. Set power wavelength to 780 nm (on power meter)
4. Zero power (on power meter)
5. Using Inspector software, click hardware configure XY-scanner settings move to, then close configure window, enter the correct center positions, i.e. 80, -30, and press enter.
6. In EOM window, change EOM 1 to always on.
7. In TriMScope window, change shutter status to open (by clicking “close”)

8. Read power from power meter.

9. In the TriMScope window, adjust the power% slider until you get the desired laser power reading (20-40 mW) from the power meter.

10. In TriMScope window, change shutter status to closed (by clicking “open”)

11. In EOM window, return EOM 1 to auto.

Placing filters

1. Unscrew two black screws and remove plate covering filters.

2. Select appropriate filters (377/50, 460/80) and dichroics (405, 505) from filter box.

3. To put a filter in a mount (25mm) drop the filter into the mount and tighten the set screw on the side of the mount.

4. Place each mount component into the correct position using the screw provided as a handle.

5. Replace the cover plate and black screws.

Taking image stacks

1. Center sample under objective.

2. Move stage to appropriate height using large wheel to the left of the stage.

3. Set filter turret position to 1 and shutter switch to “laser scan”.

4. Ensure there is enough water on the sample to make contact with the objective (if using water immersion objective)

5. Turn on transmission light.
6. Focus on the surface of the sample by turning the large focus knob (small focus does not work).

7. Turn off transmission light (and any other lights in the box).

8. Open slider to PMTs and close US-Port slider.

9. Turn on PMTs

10. Close door to enclosure.

11. In Inspector software, start video and use the joystick or mouse wheel to find the start position (top of the sample).

12. In xyz-table Z window select “set as zero” then at start position click “take”.

13. Use mouse wheel or joystick to move deeper into sample (to maximum imaging depth) then click “take” for end position.

14. Stop the video.

15. In the xyz-table window set the appropriate step size.

16. Double check that the start and end positions and step size are correct.

17. In the xy-scanner window, select appropriate frequency and line averaging for image stack. (200 frequency, 1 line average).

18. Press the start button.

19. Once recording the stack is complete, save the data by going to file→save as (save with .msr).

20. After .msr file has been saved, the data can be exported as a .tiff by clicking on the “export as tiff” button in the menu bar at the top of the software.
21. Use the joystick to move around the sample and repeat steps to record and save additional stacks.

22. Before turning on any lights make sure to close the slider to the PMTs and turn off the PMTs.

23. Taking image stacks of thick samples

24. Close sliders to PMT.


26. Turn on fluorescence light source for epi-illumination and set to low light level.

27. Turn filter turret to position 6 and flip shutter switch away from “laser scan.”

28. Focus on sample.

29. Turn off fluorescent light source.

30. Return filter turret to position 1 and flip shutter switch to “laser scan.”

31. Turn off all lights.


33. Open slider to PMTs, turn on PMTs.

34. Close door to enclosure.

35. Take images (start with step 40).

Shut down & Clean up

1. Ensure slider to PMT is closed.

2. Turn off PMTs.

3. Set PMT gains to zero and laser power to 1%. 
4. Switch laser to “stand by.”

5. Turn off all light sources.

6. Turn off laser warning light.

7. Turn off power strip.

8. Dampen lens tissue with isopropanol and blot objective lens.

9. Clean up the microscope stage and surrounding area.
C.4 Standard operating procedure for the SS-OCT1050

SOP# B02-009

Supplies

1. Sample

Equipment

2. Thorlabs SS1050 OCT system

Procedures

Starting and Operating the System

3. Turn the “POWER” switch to the “|” position.
4. Verify that the ‘POWER ON’ indicator on the Imaging Module is also turned on.
5. Depress the “LASER ENABLE” keypad.
6. Turn on the computer and monitor.
7. Start the software
8. Insert a sample under the OCT head.
9. Depress the “AIM ENABLE” keypad
10. Adjust the height to approximately 8 mm using the focusing knobs to the rear of the head.
11. Click the “1D Mode” in the OCT control panel.
12. Use the focus knob to adjust the peak signal to the left side of the screen
13. Depress the “PeakFind” button to set the OCT signal peak to 0 dB

14. Input a value into “Dynamic range (dB)” to set the minimum signal level

15. Click on the “2D Mode” tab, as shown in Figure 1.

16. Click the green triangle ‘Play OCT’ button

17. Use the Allen wrench adjust the “OPTICAL DELAY” and “POLARIZATION” if necessary.

18. Click the camera “Play CCD” tool button to open the camera window.

19. Enter appropriate values into the “2D imaging setup” control group

20. Adjust the properties in the “Image display control” control group for a good image.

21. Click on the “Spectral shaping” tool button

22. Click the “3D Mode” tab to go to the 3D imaging mode.

23. Enter the desired parameters in the window.

24. Click on the green triangle “Play OCT” button to start the 3D imaging mode.

25. Save any important data.

Exporting Data

1. Open the “3D Mode” tab.

2. Open the file containing the volume you would like to export.

3. Once the file has loaded select the box next to the plane you would like to export (XY, XZ or YZ).
4. Move the slider to the maximum position for the plane you want to export. Put all other sliders to zero.

5. Go to file → export data.

6. Select a folder or create a folder to export to and type in the file name (when saved the images will be numbered consecutively, i.e. XY1, XY2, XY3…) and click save.

Shutting Down the System

1. Close the program software. Shut down the PC

2. Depress the “LASER ENABLE” keypad to turn the laser off.

3. Depress the “AIM ENABLE” keypad to turn the aiming laser off.

4. Turn the “POWER” switch to the “0” position.
C.5 Standard operating procedure for co-localization of SS-OCT and MPM

SOP# B14-004

Supplies

1. Transfer plate (may include nose cone and heating pad)
2. Transfer plate slider
3. 2 Fasteners (bolts that fit the microscope stage)
4. Paper target

Equipment

1. SS1050 OCT system
2. Multiphoton intravital microscope
3. Multiphoton stage

Protocols Referenced

1. SS1050 OCT setup/calibration, B02-008b
2. Multiphoton setup/calibration, B02-009b

Procedures

1. Place a large post in the multiphoton enclosure to the right-rear of the multiphoton stage.
2. Attach the OCT head to the post such that it hangs over the right side of the multiphoton stage.
3. Place transfer plate on transfer plate slider.

4. Place the transfer plate assembly on the multiphoton stage such that the transfer plate is toward the back of the enclosure and the slider is toward the front of the enclosure.

5. Slide the transfer plate all the way to the left.

6. The “X” on the transfer plate marks the approximate location of the ovary (where the ovary will be). Center this “X” under the multiphoton objective.

7. Attach the transfer plate slider to the multiphoton stage by putting the bolts through the holes on each side of the slider and screwing them into corresponding threaded holes on the multiphoton stage (MAKE SURE YOU ARE USING THE CORRECT BOLT SIZE AND DO NOT OVER TIGHTEN).

8. Follow protocol for SSOCT setup/calibration, B02-008b.


10. Slide the transfer plate all the way to the left and secure the paper target to the transfer plate at the approximate location of the ovary.

11. Using the multiphoton eyepiece (follow B02-009b for thick samples) focus on the paper target.

12. Move the left set-screw or multiphoton stage until the target is in the center of the field of view.

13. Using the multiphoton, take an image of the centered target and save.

14. Without moving the target, slide the transfer plate all the way to the right.

15. Scan with the OCT to find the target.
16. Move the right set-screw and/or OCT head until the target is in the center of the field of view of the OCT image volume.

17. Once the target is in the center of the field of view, lock the OCT head in place and do not touch the right set-screw.

18. Scan and save an OCT volume to confirm the target is centered. Now the OCT and multiphoton are set for co-localized image centers.

19. Commence imaging.
C.6 Standard operating procedure for Ketamine/Xylazine anesthetic for IP injection

(80-100mg ketamine + 10mg/kg xylazine)

SOP# B07-010b

**Supplies**

1. Gloves
2. 2 sterile saline, USP, bottles with septate top
3. Ketamine (100mg/ml) solution
4. Xylazine (20mg/ml) solution
5. Syringes and needles

**Procedures**

1. Put on a pair of gloves
2. Completely empty one bottle of saline using a syringe. This will be your new Ketamine/Xylazine container.
3. Add 1166.5 µl Ketamine 100mg/ml stock and 583.25 µl Xylazine 20mg/ml stock to vial
4. Add 8250.25 µl Saline to vial
5. Gently mix.
6. Label bottle with the following information
7. 80-100mg/ kg Ketamine +10mg/kg Xylazine, date and initials
8. Fill syringe to appropriate level based on mouse weight from table below. If entire procedure is less than an hour, administer a ½ dose.

9. Using dominant hand, pick up mouse by tail and set mouse on wire cage.

10. Using thumb and forefinger of non-dominant hand grab scruff of mouse’s neck and turn mouse upside down at a slight tilt (abdomen facing up and head towards ground).

11. Use pinky finger of non-dominant hand to hold tail against palm and keep the abdomen taught.

12. Using the dominant hand, insert the needle through the skin and peritoneum and slightly pull back on syringe to insure you have not hit a blood vessel. If no blood enters needle then slowly inject dose.

13. Release the mouse into an empty cage and give 5-10 minutes for anesthesia to take effect.

Table C.3. Ketamine/Xylazine dose (and half-dose) listed by mouse weight

<table>
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<tr>
<th>Mouse weight (grams)</th>
<th>Full dose (ml)</th>
<th>1/2dose (ml)</th>
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C.7 Standard operating procedure for rodent surgery

SOP# B07-020

Supplies

1. Stainless Surgical instruments (scissors, forceps, needle holder, etc.)
2. Stainless dishes
3. Cautery pen
4. Gauze, cotton pads
5. Instrument tray
6. Hand scrub and brush
7. Hand towels
8. Bonnet
9. Surgical mask
10. Surgical gown (optional)
11. Surgical gloves
12. Shoe covers (optional)

Procedures

1. Instrument sterilization and set up
2. Surgery must be performed in dedicated area separate from animal preparation.
3. Set aside all supplies that must be sterile (any supply making contact with the animal or sterile field). *It is always useful to have two of everything in case something is accidentally contaminated during the set up to surgery.

4. Make sure all instruments are clean and rust free.

5. Place instruments in to individual sterilization packets.

6. Write date, instrument name and initials on outside of packets.

7. Insure sterilization indicator reads not sterile.

8. Repeat steps 2-4 for gauze, surgical gowns, hand towels, drapes and other autoclavable materials.

9. Sterilize all autoclavable materials in the autoclave for 1 hour gravity cycle (must be used within two weeks or re-sterilized within two weeks of use).

10. After autoclaved, insure sterilization indicator has changed to sterile.

11. Clean working surfaces in surgical area thoroughly with 10% bleach.

12. Place clean diaper pads on surfaces.

13. Place shaved, clean (providone or betadine solution followed by 70% isopropyl alcohol), anesthetized animal in heated surgical bed (follow specific surgical protocol for animal prep and follow proper anesthesia protocol for procedure [B07-010c for Ketamine/Xylazine or B07-16b for Isoflurane]).

14. Scrub animal a final time with iodine followed providone or betadine solution followed by 70% isopropyl alcohol.

15. Follow surgical attire section below before continuing to step 15.
16. A non-sterile person can unwrap the packaging on one side of the tray drape and extend the sterile tray drape to the surgeon.

17. The surgeon can take the tray drape, without touching the packaging, open it up and place it on the tray.

18. The non-sterile person can open all remaining sterile supplies by opening the sterilization packets with the opening facing away from them and towards the sterile tray and drop the sterile contents onto the sterile tray.

19. The surgeon can then organize the sterile supplies on the sterile tray drape.

20. If sterile liquids, such as saline, are needed, a sterile dish should be set up on the tray drape by the surgeon and a non-sterile person can open the container containing the sterile saline (the container is generally not sterile) and pour the liquid into the sterile dish (without touching the dish).

21. The animal drape should be dropped out onto the sterile tray and placed on the animal by the surgeon.

22. Once the supplies are unpackaged and in order and the animal is draped and under surgical level of anesthesia (confirmed by toe pinch), the surgery can commence.

23. Throughout surgical procedure the animal should be monitored at a minimum of every 15 minutes to ensure the appropriate depth of anesthesia is maintained (toe pinch [shouldn’t produce withdrawal reaction], respiratory rate [should be slow, but not too slow], mucous membranes/coloring [should be pink not white, blue or red]).
24. If the animal moves during anytime during surgery or if the anesthesia level is found to be inappropriate, the surgery must be temporarily stopped until appropriate depth of anesthesia is regained.

Surgical Attire & Hand Washing

25. Remove jewelry from hands and wrists.


27. Put bonnet so that all hair is covered. (men with facial hair should wear sideburn/beard covers).

28. Put on surgical mask.

29. Open packaging for hand towel, gloves and gown and set them on clean surface (still in packaging) without touching the sterile contents.

30. Turn on sink.

31. Open hand scrub and use brush to scrub hands, palms, under fingernails and forearms thoroughly.

32. Rinse scrub from hands and forearms, keeping hands higher than forearms such that all water drips down to elbows and not onto hands.

33. YOUR HANDS ARE NOW CLEAN

34. If hands are needed to turn off water have someone else turn it off.

35. Take hand towel out of packaging (without touching packaging) and dry hands going from one side of the towel to the other so that no part of the towel is reused.
36. Throw away the towel.

37. Take gown out of packaging (without touching packaging) and open gown.

38. Put arms through sleeves so that fingers just peek out. Have someone close the top back of the gown.

39. To tie the waist, hold the short tie and have someone else hold the paper around the other tie.

40. Turn in a circle so that the tie wraps all the way around your waist, then tug the tie out of the paper.

41. Tie the two ties together.

42. Remove the first glove from the packaging by only touching the inside of the glove and pull the glove over your hand and over the sleeve of the gown as high up as the glove will go.

43. With the gloved hand remove the other glove from the packaging only touching the outside of the second glove with the first glove and put it on the second hand, also pulling it up over the gown sleeve.

44. Return to step 14 of instrument set up to finish setting up.

When sterility is broken at any time during set up or surgery

1. Remove all non-sterile supplies including drapes they may have touched.

2. If the surgeon broke sterile field, the surgeon must rescrub and put on a new gown and gloves.
3. All drapes and instruments necessary should be replaced with sterile equipment using the above methods.

4. When performing surgery on multiple animals

5. Gloves and blades must be changed after every animal.

6. Other instruments must be cleaned and re-sterilized (bead sterilizer OK) after every animal.

Animal Recovery

1. Place animal in clean, empty cage (no food, bedding or other animals) on warming pad and observe animal until animal is ambulatory.

2. Once animal is ambulatory it can be placed in a normal clean cage with food.

3. Address post-operative pain as approved in IACUC protocol.

4. Replace fluids IP or SC if needed (0.5-1mL for mice, 3-5mL for rats).

5. Following surgery, monitor appetite, wound healing, energy and movement.

6. Remove skin closure materials at 10-14 days post-surgery.

Clean up

1. Place all sharps (needles, blades, etc.) into a sharps container.

2. Remove any biohazard waste (including gloves and gowns) to the biohazard container.

3. Decontaminate surfaces as appropriate.

4. Clean instruments with soapy water, rinse with distilled water, dry and return to storage location.
C.8 Standard operating procedure for *in vivo* mouse ovary imaging with the SS-OCT and MPM

SOP# B07-18b

**Prep/Recovery Supplies**

1. Gloves/other PPE
2. Tabletop diapers
3. Blue plastic container
4. Scale (0.1g resolution, 100g capacity)
5. 1 ml 25 5/8 gauge syringes (at least 2 per mouse)
6. Ketamine/Xylazine mix
7. Empty cage (2) [no ingestible food or bedding]
8. Heating pad
9. Hair clippers
10. Nair
11. Gauze or cotton pads
12. Distilled water
13. Iodine scrub
14. 70% ethanol
15. Heating transfer plate (with nose cone attached and fasteners)
16. Isoflurane
17. Ampicillin antibiotics
Surgical Supplies (all sterile)

1. Proper surgical attire
2. Hand scrub
3. Tray drape
4. Surgical drapes
5. Gauze
6. Saline
7. Serrated forceps
8. Scissors, iris
9. Needle holder
10. Small retractor
11. Ovary spoon and attachment (attaches to transfer plate)
12. Cautery pen (with spare tips and batteries)
13. Small round coverslip (~4 mm diameter)
14. Stainless wires
15. Rubber bands (small)
16. 4/0 chromic sutures
17. Skin staples

Equipment

1. SS1050 OCT system
2. Multiphoton intravital microscope
3. Small animal inhalation anesthetic machine

Protocols Referenced

1. SS1050 OCT setup/calibration, B02-008b
2. Multiphoton setup/calibration, B02-009b
3. Co-localization of OCT and MPM images, B14-004
4. Small animal anesthesia machine, B07-016b
5. Ketamine/Xylazine anesthetic protocol, B07-010b
6. Rodent Surgery, B07-020

Procedures

Equipment Preparation

1. Follow protocol for SS1050 OCT setup/calibration, B02-008.
2. Follow protocol for multiphoton setup/calibration, B02-009.
3. Follow protocol for co-localization of OCT and MPM images.
4. Follow protocol for set-up/use of the small animal anesthesia machine, B07-016b.
5. Set up prep area with all supplies needed.
6. Prepare surgical area by placing all necessary instruments within reach (keep in sterile packaging).
7. Place heating transfer plate in surgical area and plug in (temperature set low to medium).
8. Prepare a recovery area where mouse can lay in empty cage on a heating pad at lowest heat setting while it recovers from anesthesia.

Surgical preparation

1. Put on PPE
2. Cover working surfaces with tabletop diapers.
3. Place empty blue plastic container on scale and turn on and tare the scale.
4. Place mouse inside container on the scale and record mouse’s weight.
5. Follow Ketamine/Xylazine anesthetic protocol, B07-010b, to anesthetize mouse with Ketamine/Xylazine mix using appropriate dose for weight.
6. Place mouse in empty cage on heating pad (low heat) and allow 5-10 minutes for anesthetic to take effect.
7. Shave hair from hind limb to ribs on right dorsal side of body.
8. Treat skin with Nair for 30 seconds and gently remove Nair and remaining hair with gauze or cotton pads.
9. Rinse skin well with distilled water to remove all hair fragments and Nair residue.
10. Use providone scrub on surface of skin and bordering hair.
11. Rinse skin with 70% isopropyl alcohol.
12. Repeat steps 10&11 two more times.
13. Transfer mouse from prep area to heating transfer plate in surgical area.
14. Place mouse’s head in nose cone and start flow of Isoflurane, following protocol B07-016b.
15. Surgeon should prepare for surgery, following protocol B07-020.

16. Drape mouse using sterile drape with hole in drape located over incision site.

17. Prepare all surgical instruments following protocol B07-020.

Surgery

1. Confirm surgical level of anesthesia using tow pinch (non-sterile person!).
2. Pick up slack skin anterior to the right leg slightly above the midline, with serrated forceps.
3. Make a small horizontal incision with medium point scissors above forceps, ensuring that the incision only penetrates the skin.
4. Blunt dissect between the skin and peritoneal/muscle layer using needle holders (or other blunt instrument).
5. Pick up muscle layer with serrated forceps and make a small incision through the muscle with the scissors ensuring that abdominal contents are not damaged.
6. Retract the peritoneum, and skin to expose abdominal contents.
7. Secure sterile spoon to transfer plate.
8. Find the ovary in the fat pad and exteriorize onto the surface of the mouse.
9. Use gauze and cautery pen as needed to stop bleeding.
10. Place ovary on spoon and cover with coverslip.
11. Secure coverslip and ovary to the spoon using rubber bands or wires (with gentle pressure).

Imaging
1. Transport the mouse, transfer plate, and anesthesia machine to the multiphoton room.

2. Place the transfer plate on the stage and secure it to the rail using appropriate fasteners.

3. Follow protocols for OCT and MPM imaging and co-localization.

4. While images are being taken, record mouse number, age, weight, the date, computer file names, and your initials in lab notebook.

5. Continually assess level of anesthesia by monitoring mouse color and respiration.

If mouse stirs during image acquisition:

1. Pause current imaging program.

2. Increase the level of Isoflurane to 2+%

3. Re-assess animal and if anesthesia is at appropriate level, resume imaging.

4. During observation, periodically add several drops of sterile saline to avoid drying out the tissue.

When imaging is complete, return the mouse, transfer plate and anesthesia machine to the animal room.

Closing the surgery

1. If the surgeon broke sterility during the imaging procedure, follow protocol rodent surgery protocol about re-sterilization before continuing.
2. Release the ovary from the spoon and return the ovary to the abdominal cavity in proper anatomic position.

3. Return the peritoneum and muscle to correct positions and suture the two together using 4/0 chromic gut sutures.

4. Return the skin, oppose the edges and close with small skin staples.

Recovery

1. Dose with antibiotics for appropriate weight (20-100 mg/kg subcutaneous)

2. Place animal into an empty cage over warming pad on low heat setting.

3. Allow the animal to rest and monitor breathing, heart beat and color during recovery every 15 minutes until awake and active.

4. Once the animal has sufficiently recovered (able to ambulate and appears to be alert and active), transfer to a normal cage with bedding and food and return to the animal care facility.

5. Replace cage water with ibuprofen water 0.2 mg/ml (for two days).

Clean up

1. Remove any biohazard waste to the biohazard container

2. Clean the microscope room and animal room, decontaminating surfaces with ethanol as appropriate.

3. Clean instruments with soapy water, rinse with distilled water, dry and place into bags for sterilization.
C.9 VPR Approval #1 for Animal Subjects

The University of Arizona
TUCSON ARIZONA

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 – USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use.
The following listed proposal has been granted Authorization to Commence according to the review policies of
the IACUC:

PRINCIPAL INVESTIGATOR/DEPARTMENT:  Dr. Patricia Hoyer - Physiology

PROTOCOL CONTROL NUMBER/TITLE:
#07-037 - "Ovarian Cancer in Rats"

ACTIVE AUTHORIZATION PERIOD*:

INITIATION DATE: May 23, 2007
EXPIRATION DATE: May 22, 2010

* Projects scheduled to continue longer than the originally approved 3-year authorization period, will require the submission of a new protocol
proposal to undergo full review. Following IACUC review, a new Protocol Control Number and Authorization Period will be assigned to commence
immediately following the original expiration date.

GRANTING AGENCY: NIH

REVIEW INFORMATION:
SUBMISSION DATE: March 29, 2007
FINAL REVIEW DATE: May 23, 2007

RESULTS OF GRANT TO PROTOCOL REVIEW:
☐ No Significant Discrepancies noted  ☐ Discrepancies noted below  ☐ Not Applicable:

REVISIONS/MINORITY OPINIONS (if any):

Institutional Official: Leslie P. Tolbert, Ph.D
Vice President for Research

AUTHORIZATION STATUS FOR THIS PROJECT WAS CONFIRMED ON: May 24, 2007

Approval of this protocol does not guarantee approval of subsequent experimental protocols planned for these animals.
This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments,
and any supplemental information contained in the file noted as reviewed and approved by the IACUC.
C.10 VPR Approval #2 for Animal Subjects
C.11 VPR Approval #3 for Animal Subjects

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 – USDA No. 66-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted Authorization to Commence according to the review policies of the IACUC:

PRINCIPAL INVESTIGATOR/DEPARTMENT: Jennifer Barton
Department of Biomedical Engineering

_PROTOCOL CONTROL NUMBER/TITLE:_
#10-148 "Optical Imaging of Ovarian Carcinogenesis in a Mouse Menopause Model"

_ACTIVE AUTHORIZATION PERIOD:_
INITIATION DATE: April 22, 2010
EXPIRATION DATE: April 22, 2013

*Projects scheduled to continue longer than the originally approved 3-year authorization period, will require the submission of a new protocol proposal to undergo full review. Following IACUC review, a new Protocol Control Number and Authorization Period will be assigned to commence immediately following the original expiration date.

GRANTING AGENCY: NIH

REVIEW INFORMATION:
SUBMISSION DATE: 02/05/10
FINAL REVIEW DATE: 04/22/10

RESULTS OF GRANT to PROTOCOL REVIEW:
☐ No Significant Discrepancies Noted ☐ Discrepancies noted below ☐ Not Applicable ☐ No Grant Provided

The grant application that accompanied the protocol was for rats but this protocol is for mice.

REVISIONS/MINORITY OPINIONS (if any):

Institutional Official: Leslie P. Toibert, PhD
Vice President for Research

AUTHORIZATION STATUS FOR THIS PROJECT WAS CONFIRMED ON: April 22, 2010
Approval of this protocol does not guarantee approval of subsequent experimental protocols planned for these animals. This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.

Arizona’s First University – Since 1885
APPENDIX D: COMMERCIALIZATION POTENTIAL OF AN OCT-MPM IMAGING SYSTEM FOR OVARIAN CANCER SCREENING

D.1 Commercialization Potential of an OCT-MPM Imaging System for Ovarian Cancer Screening

The following chapter is for the partial fulfillment of a minor in entrepreneurship.
Title: Commercialization Potential of an OCT-MPM Imaging System for Ovarian Cancer Screening

Clinical Need

The lethality of ovarian cancer, largely due to late diagnosis resulting from lack of adequate ovarian cancer screening tests, presents a great clinical need that should be addressed. Ovarian cancer causes 15,000 deaths per year in the United States alone. The majority of women are diagnosed with late stage disease, at which point 5-year survival rates are only 30%. Due to the current poor prognosis and frequent late stage detection, women who are at high risk are advised to undergo prophylactic salpingo-oophorectomy—a surgery in which the fallopian tubes and ovaries are removed to help prevent formation of ovarian cancer. Oophorectomy (removal of the ovaries) is not ideal due to the risks associated with surgery, including blood loss, injury to other organs, abdominal adhesions (frequently leading to bowel obstructions) and infection. In addition to the surgical risks, oophorectomy can be devastating for pre-menopausal women due to the significant morbidity associated with early menopause (resulting from the cessation of hormone production subsequent to ovary removal). These morbidities include sexual effects (vaginal dryness, dyspareunia [pain during sex], decreased libido, and decreased sexual pleasure), vasomotor symptoms (hot flashes, hot and cold sweats), osteopenia, osteoporosis, cardiovascular disease and decreases in cognitive function (Finch 2012). The sexual side effects and vasomotor symptoms can be quite devastating to quality of life and the other health effects can be deadly. The resulting osteoporosis has been shown
to be quite severe, resulting in up to 20% reduction in trabecular bone after surgery, leading to frequent bone fractures (Cann 1980). The occurrence of cardiovascular disease results in an increased incidence of heart disease and stroke. Further, all of these changes are seen in greater severity in surgical induced menopause (oophorectomy) compared to natural menopause, as evidenced by the increase in morbidity seen after oophorectomy in women who were already post-menopausal at the time of surgery. There are options to reduce these morbidities such as hormone replacement therapy and increasing calcium intake. However, these treatments do not eliminate the symptoms and these women generally die at earlier ages than the general population due to early onset (especially for pre-menopausal women) of cardiovascular disease (Madalinska 2006).

Aside from the associated morbidities and early fatality resulting from oophorectomy, removing the ovaries and fallopian tubes greatly reduces risk, but does not completely eliminate the possibility of getting ovarian cancer. In fact, studies have shown that salpingo-oophorectomy is only 75-90% effective at preventing ovarian cancer because ovarian cancer can develop from epithelial cells left behind, other reproductive organs and the peritoneum (Finch 2012). Thus, many women still get ovarian cancer after having their ovaries and fallopian tubes removed.

Yet another reason that prophylactic oophorectomy should not be taken lightly is that only a fraction of high risk women go on to develop ovarian cancer in their lifetime. Further, although the risk is present at any time in life, the majority of women get ovarian cancer after menopause. For women with BRCA mutations, risk of developing ovarian
cancer goes up to 3% by age 40, 21% by age 50, 40% at 60 and 54% at age 80 (King 2003, Finch 2012). Therefore, it would be ideal to have a screening test that would allow a woman to keep her ovaries as long as possible (as long as no cancer is present). However, this would require screening starting at earlier ages, since as of yet there is no way to know at which age the cancer may develop. With a successful screening test, not only would women be able to prolong time to oophorectomy, but also some would be able to avoid oophorectomy all together.

Risk/benefit and Adoption

Risk of developing ovarian cancer for women in the general population is only 1.4%. When incidence of disease is very low, screening tests are not recommended because false positives can cause many unnecessary surgeries, resulting in undo costs and stress (for the patient and hospital). In the high risk population (where the risk is as much as 54% for BRCA mutation carriers), a screening test is absolutely essential to reduce stress (stemming from fear of late stage diagnosis) and costs associated with care for late stage diagnosis. Due to the fatality of ovarian cancer at late stages, sensitivity is far more important than specificity (better to take out all the cancers and some extra) for the high-risk population. As it stands, there are many unnecessary surgeries due to the practice of prophylactic oophorectomy. Thus, any screening test that keeps sensitivity where it is (75-90% for prophylactic oophorectomy) and reduces false positives (increases specificity) would be an improvement over the current method. Of course, ideally sensitivity and specificity would be near 100%.
Doctors and patients are both looking for the same outcome, high sensitivity and specificity. However, even with an accurate test there will be reluctance to adopt an invasive screening test. Due to the location of the ovaries and the high resolution necessary to visualize early cancerous changes in the ovary, it is almost certain that a screening test, particularly using imaging, will be somewhat invasive. Invasiveness of the screening test, however, will be similar to or less than invasiveness of the current laparoscopic salpingo-oophorectomy. When convincing doctors and patients to adopt a new screening method, it will be important to emphasize risk-reduction and benefits over current detection and prevention methods.

Many women are willing to undergo the significant risks of surgery and morbidity associated with oophorectomy for peace of mind that they will not get cancer and die sooner than they will from the associated morbidities. Currently, 60-90% of BRCA carriers decide to undergo prophylactic salpingo-oophorectomy, with higher percentages for post-menopausal women (Finch 2012). If the screening test is proven to be as effective at catching early cancers as oophorectomy, then these women are likely to choose the screening test, which would have significantly less associated morbidity. In addition, it is assumed that some women who elected not to choose prophylactic oophorectomy due to its associated risk and morbidities would be more likely to agree to a screening test that does not require oophorectomy.

Even if doctors and patients adopt the screening test, two important decisions must be weighed for risk vs. benefit. First, how early should screening start and second, how
frequent should screening occur. Since there is a large increase in ovarian cancers detected at age 40 in high-risk women, just before 40 would be a good age to start screening. In fact, studies have shown that 2.5-18% of high-risk women are found to have early stage cancer at the time of preventative oophorectomy (not clinically detected before oophorectomy) when oophorectomy was performed between 30-65 years old with an average of 48 years old (Colgan 2001, Powell 2005). Therefore, beginning screening at the age oophorectomy is currently recommended (35 years of age for high-risk women) would be acceptable for most patients. However, women who have a family history of ovarian cancers at an early age should begin screening at an even younger age.

Since little is known about the time required for ovarian cancer progression, additional experiments will need to be done to determine the frequency of testing. However, the necessary frequency can be estimated based on some previous studies. For example, one model for ovarian cancer development predicted that ovarian cancers spend on average 4 years in situ as stage I or II and 1 year as stage III or IV before they are clinically apparent (Brown 2009). Thus, screening would need to be performed at least every 4 years. With proper screening initiation and frequency it is likely that all ovarian cancers can be found at stage one disease, potentially increasing overall five year survival to 90%.

Market Size

BRCA mutations have a prevalence of 0.5-8.3%, depending on the race/ethnicity of the population (highest for Hispanic and Ashkenazi Jewish decent) (John 2007). Around 2%
of the general population is expected to have BRCA mutations. There are approximately 150 million women in the United States, leading to approximately 3 million women who are BRCA carriers. However, the women that are more likely to be screened are those at highest risk (older ages). Since there is a steep incline in risk at 40 years of age, it would be recommended that high-risk women over 40 be screened. This puts the actual market size at approximately 1.2 million (BRCA carriers over 45 years old estimated from US census 2010) (Howden 2011).

However, only 10-15% of ovarian cancers are in BRCA positive women (cancer.gov). With the development of a good screening test, other high risk women with other genetic mutations/family history of cancer may also adopt the use of a screening test and further increase the market size.

Current State/Competitive comparison

Currently, no screening tests exist because all past attempts at screening have been found to have too low performance and are not recommended for clinical use. The methods previously tested, without success, include ultrasound imaging and CA-125 blood serum testing. Both of these methods resulted in many false positives. The majority of adnexal masses found on ultrasound are benign and frequently resolve within 6 months of initial detection. One ultrasound study estimated that in screening of 100,000 women above the age of 45, ultrasound would correctly detect 40 cancers, and incorrectly diagnose 5,400 non-cancers as cancer (Campbell 1988). CA-125 is frequently elevated due to conditions other than ovarian cancer (other cancers, lifestyle). Furthermore, a number of ovarian
cancers (especially early stage) do not result in high CA-125 serum levels and are not detected in blood tests. Due to the failure in detecting cancers, the frequent false positives, and the overall lack to reduce mortality, neither of these methods is recommended for screening purposes (Buys 2011, Teneriello 1995).

There are no current competitors for ovarian cancer screening. However, many researchers are searching for imaging methods that can detect early ovarian cancer so there are likely to be new screening methods developed in the near future. The optimal method will likely be determined by the cost-benefit ratios.

Technology: Advantages and Challenges

The technology used in the studies described in this dissertation included optical coherence tomography (OCT) and multiphoton microscopy (MPM). Both OCT and MPM are optical imaging methods that use light to interrogate the microscopic structure of the tissue. The back-reflected or fluorescent light from these systems is collected over a volume to give 2D and 3D information about the tissue. The collected data can be analyzed in a number of ways. In these studies, analysis was primarily morphologically based, but other analysis methods, such as relative intensity values may provide additional useful information. The sensitivity and specificity were high for the quantitative method of analyzing collagen structure using texture and frequency parameters. Further, all imaging techniques showed microscopic, early changes that would not be visible with other clinically used imaging systems such as ultrasound.
Studies have shown that for most of the occult period, serous cancers are less than 1 cm diameter (Brown 2009). With the high resolution of this method to detect early cancerous changes in ovaries less than 2 mm in size, it is highly likely an OCT-MPM system will be able to detect development of cancers that are less than 1 cm in size. Thus, there is great promise for using these technologies for ovarian cancer screening in the future.

In addition to the proven utility in visualizing early cancerous changes, these technologies have the advantage that they can be implemented minimally invasively for in situ imaging such that no tissue has to be removed. Furthermore, these technologies do not damage the tissue during the imaging process. OCT has additional advantages that it is inexpensive and portable. Further, OCT is already approved for clinical use (imaging the eye) and is currently being investigated for multiple other applications, so it is likely to gain FDA approval for new indications. MPM is not currently approved for clinical use and has pitfalls of being large and quite expensive due to the cost of the laser, but advancements in laser technology may bring the price down in the future. Combining OCT with MPM would be advantageous in providing additional diagnostic information due to the ability of MPM to get high resolution images and record multiple spectrally-separated phenomena at one time.

However, there are some technical challenges that will need to be addressed before an OCT-MPM system can be made clinically available. The main technical challenge is designing and building a probe to reach the ovary. The systems used in this study were table top microscope set ups. For imaging in a human they would need to be packaged
into a smaller portable system with a probe that can reach the ovary in a minimally invasive manner (not open surgery as was performed on the mice in this study). There are at least three possibilities for the design of an imaging probe that can reach the ovary for imaging: transvaginal, laparoscopic, and endoscopic probes. A transvaginal probe would be used similarly to the transvaginal needles used in collecting eggs for in vitro fertilization. In this procedure, a small needle is put through the vaginal wall, into the abdominal cavity and guided to the surface of the ovary. This procedure is relatively simple and results in little discomfort for the patient, but would make it difficult to interrogate all surfaces of the ovary and fallopian tube because there is minimal range of motion. The laparoscopic method would be similar to what is currently done during oophorectomy, up until the point of ovary removal. This would allow easy visualization of the ovaries and fallopian tubes but is the most invasive option. The transvaginal and laparoscopic probes would be enhanced with an articulating tip to look at the fallopian tube. Lastly, an endoscopic method would use a small diameter endoscope/falloposcope, threaded through the cervix, uterus and fallopian tubes to reach the ovary. This would allow visualization of the inside of the fallopian tubes (not provided by other methods) and the ovarian surface, but will have the most technical challenges to overcome. The size and flexibility requirements for each of these probes are described in table D.1.

Table D.1. OCT-MPM probe size and flexibility requirements.

<table>
<thead>
<tr>
<th>Probe Type</th>
<th>Diameter</th>
<th>Length</th>
<th>Flexibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laparoscope</td>
<td>5-10 mm</td>
<td>~35 cm</td>
<td>Rigid, articulating tip</td>
</tr>
<tr>
<td>Transvaginal</td>
<td>&lt; 2 mm</td>
<td>~32 cm</td>
<td>Rigid, articulating tip</td>
</tr>
<tr>
<td>Endoscope</td>
<td>&lt; 1 mm</td>
<td>&gt;25 cm</td>
<td>Flexible</td>
</tr>
</tbody>
</table>

The packaging of the optics required to create these types of probes presents another challenge. OCT has previously been packaged into laparoscopes for human imaging and miniature endoscopes for small animal imaging (Hariri 2009, Hariri 2010). Thus, creating laparoscopic or transvaginal probes for OCT will be possible without much difficulty. The challenge will be to make an endoscopic OCT probe small and flexible enough to reach the ovary without damaging tissue. This requires the probe to fit through 0.5 mm canal at the smallest part of the fallopian tube (Sokol 2011). The design of a 0.5 mm probe is technically feasible but the fragility is likely to make controlled use for traversing a complex geometry extremely difficult. Despite potential challenges, the overall ease to build, low cost and portability of OCT make it a valid option for clinical use as a screening method.

Due to the required high energy for multiphoton events to occur, building MPM probes will be a greater challenge than OCT. In MPM high instantaneous energy is achieved with a femtosecond pulsed laser. The quick pulses allow the multiphoton phenomena to occur, while still keeping the average power low enough so that tissue is not damaged. Dispersion that occurs in optical fibers causes these pulses to spread out so that the high instantaneous (femtosecond) pulses can no longer be achieved. Some small rigid MPM endoscopes have been built and used for in vivo imaging (Konig 2001, Konig 2007). The need for long endoscope presents a technical challenge that will be difficult to solve. This means that a laparoscopic or transvaginal method may be implemented, and a
falloposcope/endoscope is less likely. The benefits and pitfalls of OCT and MPM probes for clinical use are summarized in table D.2 below.

Table D.2. Benefits and pitfalls of OCT and MPM probes for clinical use

<table>
<thead>
<tr>
<th>Benefit/Pitfall</th>
<th>OCT</th>
<th>MPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>High resolution</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Adequate depth penetration</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Non-destructive</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>In situ imaging</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Fast imaging</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Inexpensive</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Clinically approved</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Portable</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Collects multiple phenomena</td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

MPM appears to provide enough useful information that it may be worth the effort to address the technical challenges required to make an endoscopic or laparoscopic probe for cancer screening. However, there is also an option of using other less technically complex or less expensive imaging modalities to obtain similar information to that which is obtained from multiphoton. For example, confocal fluorescence microscopy (single photon fluorescence) can be used to see endogenous fluorescence and polarized light microscopy can be used to interrogate collagen structure. Using a non-laser light source would be a great way to decrease cost of goods and make the FDA approval process easier. However, despite reduction in technical challenges by implementing less complex systems in a screening probe, development and validation would take similar time and money to complete a clinical system.

Barriers to Entry
There are many OCT systems on the market for research and clinical use. This includes systems marketed by Thorlabs, Zeiss, Optovue, Boston Scientific, Volcano, Terumo and Heidelberg Engineering. The clinical devices are primarily for retinal imaging and are configured as tabletop systems with chin and forehead rests with the scanner at eye level and are not practical for non-ocular applications. The research systems are generally handheld probes and can be used for numerous surface imaging applications. There are also MPM systems on the market that are used for research purposes. Companies offering multiphoton microscopes include Thorlabs, Zeiss and LaVision BioTec. There are currently no OCT-PM systems on the market. The main barrier to entry is the high cost of developing and validating a new system for clinical use. Once funding is available and a probe can be developed additional barriers to entry can be created. For example, it will be essential to patent new aspects of the probe and image analysis algorithms. Patents encompassing OCT and MPM technologies and endoscopic probes already exist but there is the possibility of creating new probes for the OCT-PM system that have patentable components. The patentable components may include mechanisms for movement control for guidance through the complex anatomy and components enabling longer endoscopes with MPM ability. The algorithms used in these studies were either part of pre-packaged software or implemented slight changes from previously described algorithms, so they are unlikely to be patentable. However, more advanced algorithms are likely to improve upon this study and may have patentability if they are new or differ from prior algorithms in a non-trivial way (Diamond 1981, Gottschalk 1972, Parker 1978).

Reimbursement Strategy
There is a current movement in United States healthcare towards reduced costs and sustainability of healthcare services. Due to this movement, medical groups and policy makers have called for cost-effectiveness analysis and policy is expected to change to favor outcome-based or value-based reimbursement (Weinstein 2010, Stout 2013, Carlson 2010, Garrison 2011, Hettiger 2012). Meaning, a procedure must achieve a certain outcome and cost-effectiveness in order to get reimbursed by insurance. This results in a desire to eliminate clinical practices that show no benefit and selecting prevention, detection or treatment options that are the most effective in terms of both clinical outcomes and cost-effectiveness. The overall goal of this movement is to reduce the number of unnecessary tests and other medical procedures and, subsequently, cut costs and improve health outcomes.

Owing to the goal of catching cancer early, the majority of current screening tests oversample, resulting in many unnecessary medical procedures. There is a multitude of evidence of medical doctors’ current changing attitudes and resultant reduction in screening tests. For example, in women’s health, doctors’ recommendations and insurance reimbursement for screening tests have been reduced in frequency. The recent reductions in screening frequency include cervical cancer, breast cancer and ovarian cancer. In the past, women were advised to have a pap smear every year to detect cervical cancer. In 2012, recommendations for cervical cancer screening were changed to every 3-5 years in women aged 21-65 (recommended by USPSTF). Also in the past, mammograms were recommended annually starting at age 40 to detect breast cancer. In 2009 breast cancer screening was reduced to every two years and only recommended in
women aged 50-74 (recommended by USPSTF). Recently mammograms have once again been under great scrutiny, suggesting that guidelines may change again. In the past some medical centers screened women for ovarian cancer using ultrasound and CA-125. In 2012 the recommendation was not to screen women for ovarian cancer with either of these methods (recommended by the USPSTF, ASCO, and NCCN).

In general, doctors are moving towards doing the least number of tests possible to achieve the desired result and, in the future, will likely limit imaging tests to the methods and frequencies that are shown to be most effective (Garrison 2011, Hettiger 2012). Thus, in order for any screening test to be implemented in the future it will need to be highly effective and perfectly sampled. Furthermore, it will be necessary to prove that early detection prevents cancer progression and metastasis and thereby cuts down on long term medical costs (Smith 2011).

Additionally, with the short-term cost component associated with outcomes-based medicine, the charge for the screening test should cost similar to the current detection methods. The costs for methods currently used for ovarian cancer detection and expected cost of a diagnostic laparoscopy/endoscopy are shown in table D.3. Creating a new technology is extremely expensive and would likely charge a lot per procedure to recover development costs. However, even at high cost, a screening test that works to catch early ovarian cancer is likely more economically favorable than the alternatives, which are usually a prophylactic oophorectomy with overnight hospital stay or late stage cancer treatment including surgical debulking and chemotherapy.
Table D.3. Comparison of current medical procedures for ovarian cancer (estimates from BCBS)

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oophorectomy + hospital stay</td>
<td>$40,000+</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>$1,000-3,000</td>
</tr>
<tr>
<td>CA-125 serum test</td>
<td>$175</td>
</tr>
<tr>
<td>Diagnostic laparoscopy/endoscopy (outpatient)</td>
<td>$3,000-5,000</td>
</tr>
</tbody>
</table>

*these costs represent the charges shown on the bill, not the actual cost to the hospital for doing the procedure, which is actually much lower (not accounting for capital equipment cost).

Regulatory Approval

Existing clinical OCT systems and gynecologic laparoscopic accessories are considered FDA class 2 medical devices. This includes the light source, delivery systems, scope, scanning optics and other instruments. The product classifications include the OCT Imaging System (general) (892.1560), OCT intravascular catheter (870.1200), OCT ophthalmoscope (886.1570), gynecologic laparoscope and accessories (884.1720) (FDA 2013). Since an OCT system for imaging the ovary would be substantially equivalent to predicate devices, a 510(K) would be necessary to obtain FDA approval for a gynecologic laparoscope using OCT. A 510(k) is a premarket notification made to the FDA to demonstrate the device is safe and effective and substantially equivalent to another FDA approved device. Review fees for a 510(k) are $4,960 standard and $2,480 for small businesses with less than $100 million in sales (FDA 2013). The MPM has no predicate devices that have been FDA approved, thus it most likely requires a PMA. PMA
standard fees are $248,000 or $62,000 for a small business (FDA 2013). However, the
FDA can waive fees for State and Federal entities as well as the first PMA submission for
a small business with gross sales less than $30 million (FDA 2013). FDA approval takes
approximately one year to obtain (assuming the results and conclusions of the submission
are satisfactory).

FDA approval for an OCT-MPM (going either the 510(k) or PMA route) would require a
clinical study to prove safety and effectiveness of the device. An investigational device
exemption (IDE) from the institutional review board is required prior to clinical study
and could take one year to obtain. Initial imaging studies for ovarian cancer generally
enroll 50-100 patients and take one to five years. Examples of clinical studies for ovarian
cancer detection include ultrasound (NCT00531570 [n=100], NCT00248820 [n=100],
and fluorescence imaging with folate-FITC (NCT01511055 [n=50]) (clinicaltrials.gov).

Estimated costs for a 5 year, one-hundred patient clinical study and FDA approval is at
least $1,300,000 [Table D.4]. Thus, the entire regulatory process, including IDE, clinical
study and FDA approval, is likely to take at least seven years and cost at least $1,300,000
and could actually be upwards of $2 million depending on filing fees, number of patients
enrolled and number of investigators/study sites.

Table D.4. Estimated cost components for device approval
(5-year clinical study with 100 patients).

<table>
<thead>
<tr>
<th>Item</th>
<th>Cost per Item</th>
<th>Total per Item</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical Doctors/PI (3)</td>
<td>$8750/doctor/year (NIH</td>
<td>$131,250</td>
</tr>
<tr>
<td></td>
<td>rate)</td>
<td></td>
</tr>
<tr>
<td>Technical Staff (3)</td>
<td>$30,000/year</td>
<td>$90,000</td>
</tr>
<tr>
<td>Instrument Sterilization</td>
<td>$40/patient</td>
<td>$4,000</td>
</tr>
</tbody>
</table>
### Financials

Significant costs, amounting to at least $500,000 would be needed for development of an OCT-MPM system that could actually be used in the clinical setting. Following technology development ($500,000 over 2-3 years), a clinical study with a large patient population would be completed ($1.3 million over 7 years). Thus, the total cost to get to FDA approval would likely amount to $2-3 million and 10 years’ time.

Actual cost of goods sold (COGS) for a complete OCT-MPM system would likely be near $100,000. In order to compete with pricing of other imaging modalities the selling price could not be much more than $120,000. For example, OCT ophthalmoscopes sell for $40-120k, clinical ultrasounds sell for $30-100k, CT scanners for $140k+, and X-ray mammography systems sell for $15-30k. Although the OCT-MPM system may be found to have applications for other cancers, it is most likely to start out being marketed for a single application. Thus, the best price point would be near other systems that are used for single applications, like the OCT ophthalmoscope which has about a $120,000 max sales price. Due to the high cost of the laser, it would be extremely difficult to decrease the COGS until alternate, less-expensive light sources become available. In addition to
short term revenue from capital equipment there are potential add-ons for long term
revenue. These add-ons may include maintenance, system upgrades, software upgrades
and, potentially, disposable parts.

Without having a complete clinical system if it difficult to gauge interest and know
exactly what revenues could be expected. However, it can be assumed that for a
screening test for ovarian cancer with high sensitivity and specificity, the majority of top
gynecological oncologists will want one. There are about 320 top gynecologic
oncologists at 160+ medical centers across the United States, plus more in other countries
(US News 2012). Goals for numbers of systems sold and total revenues per year can be
constructed from looking at revenues of other medical imaging system. Global revenues
from common clinical imaging modalities are shown in table D.5 (BCC Research 2013).
Compared to other devices, many of which serve many markets, $2 million seems to be
an achievable annual goal and would require selling approximately 16 devices per year.
At that pace all 160 top medical centers in the US would have devices in 10 years.
Further, at the end of that 10 year period the profits would have been $20,000(profit per
device)*160(number of devices sold)=$3.2 million, which is enough to cover the
development and approval costs (estimated at $2-3 million), but not enough to make a
profit. Thus, to actually be profitable it would be better to set the sales price to $180,000
(more expensive than any of the other clinical systems).

Table D.5. Estimated global revenues from imaging devices in 2012

<table>
<thead>
<tr>
<th>Imaging Modality</th>
<th>Revenue ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray &amp; CT</td>
<td>13 billion</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>5.6 billion</td>
</tr>
<tr>
<td>MRI</td>
<td>5.4 billion</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PET</td>
<td>2.7 billion</td>
</tr>
</tbody>
</table>

Estimating hospital revenues from conducting the screening test requires looking at the values from the number of women that should be screened (at least 1.2 million), the screening frequency (at least once every 4 years) and the estimated costs per screening procedure ($5,000). Adding up these values puts the yearly revenue from screening at $1.5 billion. However, if the adoption is not 100%, but closer to adoption of oophorectomy (60-90%), hospital revenues would be $900 million to $1.3 billion.

Summary

Ovarian cancer is a devastating disease due to frequent late stage presentation and therefore renders the need for a screening method for the more than 1.2 million women at extremely high-risk for developing cancer. In the present study, data from OCT and MPM imaging modalities has shown ability to detect cancer development in early stages in mouse ovaries. These results show that a combined OCT-MPM imaging system may be useful as a clinical screening test for ovarian cancer. Due to the fact that the technology does not yet exist in a compact, minimally invasive form, it is difficult to anticipate many factors such as clinical utility, adoption, and revenues. However, the great need for a screening option combined with the promising results for early detection and the movement of the U. S. healthcare system to outcome-based reimbursement, suggests that additional effort and funding should be put toward the development of an OCT-MPM system for cancer screening.
REFERENCES


