Applications of Digital Microfluidics For The Quantification of Small Molecules from Solid Droplet-Scale Tissue Samples

by

Sara Mohammad Anis Abdulaziz Abdulwahab

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy – Analytical Chemistry

Department of Chemistry
University of Toronto

© Copyright by Sara Abdulwahab (2017)
Applications of Digital Microfluidics For The Quantification of Small Molecules from Solid Droplet-Scale Tissue Samples

Sara Abdulwahab

Doctor of Philosophy – Analytical Chemistry

Department of Chemistry
University of Toronto
2017

Abstract

Digital microfluidics (DMF) is a microscale liquid handling technique in which discrete liquid droplets are manipulated on a two-dimensional array of hydrophobic, insulator-coated electrodes with the aid of electrodynamic forces. Because of its several advantages, including, ease of automation, portability, and ability to perform multiple sets of processes, DMF is gaining popularity for a number of chemical and biological microscale assays. Because of its channel-free nature, DMF is particularly promising for solid sample processing, enabling the incorporation of solid materials into the assay work-flow without risk of clogs.

In this thesis, I explore the capabilities of DMF as a tool for processing microscale tissue samples that are otherwise difficult to handle using macroscale tissue processing technologies. Chapter two describes the first method useful for multiplex quantification of steroid hormones in core-needle-biopsy (CNB) tissue samples. This new technique expands the breadth of information that can be obtained from the analysis of milligram-size tissue specimens when compared to previously reported macroscale tissue processing techniques. Chapter three describes an application for the method described in chapter
two – an investigation into the effect of bilateral salpingo-oophorectomy procedure (a surgical procedure routinely employed for reducing breast cancer risk) on steroid hormone levels in CNB tissue samples, pre- and post-surgery. Chapter four describes the first miniaturized platform for rapid analysis of estradiol (E2) from CNB samples directly on a DMF device. This newly developed automated, portable platform combines the strength of DMF for solid sample processing with the flexibility of magnetic bead-based immunoassays for E2 detection. Chapter five describes an extension of the techniques developed in chapters 2-3 for on-chip endometrial tissue processing. Using this system, endometrial tissue specimens were extracted, cleaned-up and quantified for nine small molecule target analytes by HPLC-MS/MS. The results obtained using the new method are preliminary, yet promising, and may in the near future present a useful new tool for functional evaluation of endometrial tissue maturation and receptivity for in-vitro-fertilization (IVF) procedures. Finally, chapter six highlights the principles and concepts derived from this work, and concludes with ideas for future applications of the DMF platforms described in this dissertation.
Dedication

This thesis is dedicated to my parents, Anis and Wedad, without your, love, kindness and continuous support I wouldn’t have been able to complete this work.
Acknowledgments

First and foremost I would like to acknowledge my supervisor Prof. Aaron R. Wheeler, to have given me the opportunity to be a part of his lab and for providing me with the guidance and the support when I most needed it. Aaron's exceptional kindness, excellent mentorship and patience are all qualities any student would seek in a supervisor. I certainly wouldn't have been able to continue this course without Aaron's input and motivation.

I would like to extend my deepest gratitude to my committee members Professors Ulrich Krull and Rebecca Jockusch for their valuable advice through the course of my doctorate, I have been extremely fortunate to have you as my supervisory committee, you have always offered me sincere advice, and pushed me to pursue a thorough understanding of my work during personal meetings, annual meetings and sitting in your classes. I would also like to thank Prof. Kagan Kerman for serving on my comprehensive oral exam committee and Dr. Joanne Kotsopoulos for serving on the committee for this thesis defense and for many insightful discussions and advices regarding my work. Special thanks to Dr. Philip Britz-McKibbin for serving as an external examiner for this thesis defense.

I would like to extend my sincere thanks to my collaborators, Dr. Robert Casper, Dr. Steven Narod, Dr. Hala Gomaa, Dr. Lucy-Ann Behan, Dr. Hend Ahmado, Dr. Tzu-Bou Hsieh, Dina Nikitina and again Dr. Joanne Kotsopoulos for their valuable contributions to the several projects that I have been involved with.

To my friends and colleagues at the Wheeler's lab, I owe many of you a great share of gratitude, particularly Dr. Joannes Hewel, Dr. Mike Watson and Dr. Nelson Lafreniere, for training me when I first joined the lab. I’d like to thank Dr. Jihye Kim for being there during difficult times, and for making the lab experience more fun. Dr. Alphonsus Ng for willingness to help, and for constantly motivating everyone to be productive. Dr. M. Dean Chamberlain for being constantly available for in-depth advice, Charis Lam for the patience you've demonstrated and for the time and effort you've put
into our work together, Cassandra Lord and Lisa Ngo for their invaluable help with all the office and paperwork, and Michal Dryden for his help with photo-taking.

I would also like to thank Dr. Jared Mudrik, Ian Swyer, Brendon Seale, Darius Rackus, Chris Dixon, Dr. Kihwan Choi, Betty Li, Dr. Ryan Fobel, Haozhong Situ, Man Ho, Alexandros Sklavounos, Julian Lamana, Christian Fobel, Dr. Ed Regan, Nooman Mufti, Jeremy Wong, Yue “Alex” Yu, Dr. Sam Au, Dr. Mohtashim Shamsi, Dr. Steve Shih and Dr. Lorenzo Gutierrez for being a group of amazing labmates.

I would like to thank Dr. Henry Lee, Yimin Zhou and Dr. Matt Forbes for their help and guidance through my time at Bahen clean room and at AIMS.

I would also like to thank Yousria Fekry, and the Farid’s (Adel, Basant and Omar) for welcoming me in town, for the amazing hospitality and for being my main source of advice during my first months in Toronto. To my friends Mona El-mosallamy, Nosayba Elsayed, Sarah Salem, Somaia Youssef, Bailsan Khashan, Nagwa Elashmawy, Toka Sabry, Aya Aboudina, Rana Morsy and Eman Hammad thank you for your exceptional support and thoughtfulness, I have always considered you more like a second family to me during my stay in Toronto.

Finally, I have to thank my family; my husband Tamer, thank you for helping me realize my strengths, for motivating me and for your support and care. Mom and dad, thank you for giving me everything that I needed, for always believing in me, for pushing me to succeed, for comforting and supporting me and for always being there when I needed you. Shady, Mohammad, Leora, Sara, Dana, Adam, Zeina and Lily, your presence in my life have certainly made me a better person.
# Table of Contents

Acknowledgments...........................................................................................................v
Table of Contents...........................................................................................................vii
Overview of Chapters......................................................................................................x
Overview of Author Contributions..................................................................................xii
List of Tables..................................................................................................................xv
List of Figures....................................................................................................................xvii
List of Abbreviations.......................................................................................................xix

1 Chapter 1 Review of Tissue Biomarker Quantification: Methods and Applications...1
   1.1 Tissue Biomarker Quantification Applications.........................................................1
      1.1.1 Tissue Histopathological Applications.............................................................1
      1.1.2 Tissue Proteomics and Metabolomics...............................................................4
         1.1.2.1 Tissue Metabolomics.................................................................................4
         1.1.2.2 Tissue Proteomics......................................................................................7
      1.1.3 Tissue Microarrays..........................................................................................10
      1.1.4 Tissue Molecular Testing...............................................................................10
      1.1.5 Mass Spectrometry Based Imaging Applications...........................................12
   1.2 Tissue Processing Procedures..................................................................................14
      1.2.1 Initial Tissue Handling Steps.............................................................................14
      1.2.2 Sample Extraction............................................................................................17
         1.2.2.1 Liquid-Liquid Extraction.........................................................................17
         1.2.2.2 Solid-Phase Extraction.............................................................................19
   1.3 Sample Detection ..................................................................................................20
      1.3.1 Mass Spectrometry.........................................................................................20
      1.3.2 Enzyme Linked Immunoassay.........................................................................22
   1.4 Microfluidics...........................................................................................................22

2 Chapter 2 A Microfluidic Technique for Quantification of Steroids in Core Needle Biopsies.................................................................................................................24
   2.1 Introduction..............................................................................................................24
   2.2 Experimental..........................................................................................................26
      2.2.1 Reagents and Materials....................................................................................26
      2.2.2 DMF Device Fabrication, Assembly, and Operation.......................................27
      2.2.3 Porous Polymer Monolith Formation.............................................................27
      2.2.4 Rat samples.....................................................................................................28
      2.2.5 Human Samples...............................................................................................28
      2.2.6 DMF Sample Processing...................................................................................29
Overview of Chapters

This thesis describes the development and implementation of techniques for the extraction, clean-up and analysis of targeted small molecules from droplet-scale tissue matrices using digital microfluidics (DMF) coupled to high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and Enzyme Linked Immunosorbent Assay (ELISA) that I developed and used while working in the Wheeler Group at the University of Toronto. Overall the theme of this work has been the evaluation of the utility of DMF for processing tissue samples; each chapter is described below.

Chapter 1 reviews the literature for the relevant topics encountered during the course of the thesis. A special emphasis is placed on tissue biomarker quantification applications and tissue processing steps.

Chapter 2 describes a method for the multiplexed extraction and quantification of steroid hormones from core-needle-biopsy samples using digital microfluidics and HPLC-MS/MS. This method represents the first technique for DMF-CNB sample processing, and includes a comparison between the DMF method performance with that of a common macro-scale tissue sample processing technique.

Chapter 3 builds on results from Chapter 2, describing an application for CNB sample processing on DMF, namely, to interrogate steroid hormone levels in the breast tissue of subjects prior to and post-surgical oophorectomy procedures. Findings from the results support evidence for the suitability of DMF as a platform for precious tissue sample analysis.

Chapter 4 describes a novel miniaturized platform for quantification of Estradiol (E2) in CNB samples using DMF. The new technique combines the power of DMF for solid sample processing with the flexibility of magnetic bead based immunoassays. The performance of the new assay was compared with that of HPLC-MS/MS, and the duration of the assay was tuned to be short (~40 min), compatible for use in a doctor’s office or small clinic for point-of-care testing and on-site decision making.
Chapter 5 presents an extension of the techniques described in chapters 2-3, applied to the processing and analysis of endometrial tissue samples, motivated by an eventual goal to develop functional assessment tools for evaluation of endometrial tissue maturity and growth. Several key tests were conducted, including the distribution of analyte consistency across different locations of the tissue and the measurement of extraction efficiency by standard addition.

Chapter 6 provides a summary of the work presented herein and suggestions for future development.
Overview of Author Contributions

The data presented in this dissertation is the result of collaborative team work that would have not been possible without the hard work from a number of individuals in the Wheeler group at the University of Toronto, and from other groups that I have been fortunate to work with. Here I outline the contributions made by myself and others towards the work presented in this thesis.

Chapter 2 describes the extraction and quantification of steroid hormones from core-needle-biopsy samples. This work represents a collaboration between the Wheeler group and the group of Prof. Robert Casper at Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto. Experimental planning and execution, and manuscript preparation was jointly carried out by me and Dr. Jihye Kim (a former post-doctoral fellow in the Wheeler group). Dr. Kihwan Choi (a former postdoctoral fellow in the Wheeler group) assisted in running human tissue sample extractions on DMF. Dr. Nelson M. Lafreniere (a former graduate student in the Wheeler group) helped with DMF device fabrication, DMF device operation, and in creating preliminary versions of figures. Dr. Jared M. Mudrik (a former graduate student in the Wheeler group) helped with porous polymer monolith fabrication. Dr. Hend Ahmado, and Dr. Lucy-Ann Behan (all formerly from the Casper group) collected the core need biopsy tissue samples used for analysis and assisted in interpretation of the results. Dr. Wheeler, Dr. Kim and I wrote the text with input from the other authors. This chapter was published in Analytical Chemistry, with co-first-authorship shared between me and Dr. Jihye Kim: Kim, J.; Abdulwahab, S.; Choi, K.; Lafreniere, N.M.; Mudrik, J.M.; Gomaa, H.; Ahmado, H.; Behan, L.-A.; Casper, R.F.; Wheeler, A.R., “A Microfluidic Technique for Quantification of Steroids in Core Needle Biopsies,” Analytical Chemistry 2015, 87, 4688-4695.

Chapter 3 describes work that was performed in collaboration between the Wheeler group, the groups of Dr. Joanne Kotsopoulos and Dr. Steven Narod at the Women's College Research Institute at the University of Toronto, and that of Dr. Casper at Mt. Sinai Hospital. The work features an application for multiplex steroid quantification of core-needle-biopsy samples for patients undergoing oophorectomy. I did the majority of
the laboratory experimental work for this chapter, including device fabrication, method optimization, processing tissue samples on DMF, and quantification by HPLC-MS/MS. Dina Nikitina (from the Kotsopoulos and Narod groups) was responsible for coordination of the study with respect to obtaining research ethics board approval, enrolling and consenting study subjects and liaising between the groups for sample collection and storage. Dr. Lucy Ann Behan (from the Casper group) assisted in patient recruitment and collected CNB samples. Dr. Kotsopoulos and I analyzed the data and wrote the text with input from the other authors. I will be the first author on a manuscript derived from this chapter, to be submitted for publication in the near future.

Chapter 4 describes an automated, miniaturized DMF platform for small molecule analysis directly in CNB samples. Dr. Alphonsus Ng (a former graduate student and postdoc in the Wheeler group) and I worked together in planning and executing the work. I did most of the device fabrication, method validation and tissue processing for this chapter. Dr. M. Dean Chamberlain (a current research associate in the Wheeler group) assisted by harvesting rat breast tissue samples. Dr. Lucy-Ann Behan, Dr. Hala Gomaa and Dr. Hend Ahmado (formerly from the Casper group) recruited patients and collected human tissue samples. This work was presented at the 19th International Conference on Miniaturized Systems for Chemistry and Life Sciences 2015: S. Abdulwahab, A. H. C. Ng, M. D. Chamberlain, LA Behan, R. F. Casper and A. R. Wheeler, “Microfluidic Platform For Quantification Of Estradiol In Core-Needle-Biopsies, MicroTAS 2015, Gyeongju, South Korea. Dr. Wheeler and I wrote the text, with input from the other authors. I will be the first author on a manuscript derived from this chapter, to be submitted for publication in the near future.

Chapter 5 describes the small-molecule analyte quantification in endometrial tissue samples using DMF for extraction and HPLC-MS/MS for analysis. The experimental work for this chapter, including device fabrication, HPLC-MS/MS method development, tissue processing on DMF and data analysis was mainly done by me. Charis Lam (a current graduate student in the Wheeler group) assisted with some of the device fabrication and sample processing, as well as with negative-mode ESI analysis. Dr. Tzu-Bou Hsieh (formerly of the Casper group) recruited patients and collected samples. Dr.
Wheeler and I wrote the text (with input from the other authors), which will (eventually) be used as the basis of a manuscript to submit for publication.
List of Tables

Table 2.1. HPLC-MS/MS with multiple reaction ion monitoring (MRM) conditions for eight deuterated and non-deuterated hormones ...............................................................31

Table 2.2. Calibration data for four steroid hormones................................................................34

Table 2.3. Measured hormone amounts in core needle biopsy (CNB) breast tissue samples from human subjects normalized by sample mass..........................................................42

Table 3.1. HPLC-MS/MS with multiple reaction ion monitoring (MRM) conditions for eight deuterated and non-deuterated hormones ..............................................................53

Table 3.2. Characteristics of women at the time of bilateral salpingo-oophorectomy, by BRCA mutation status ..............................................................55

Table 3.3. Hormone levels in core needle biopsy (CNB) of breast tissue samples among the study participants, normalized by sample mass before and after surgery........................................57

Table 3.4. Serum hormone levels among study participants before and after surgery..................58

Table 3.5. Summary of previously published hormone concentrations in non-malignant breast tissue samples from premenopausal human subjects normalized by sample..........................68

Table 3.6. Summary of previously published hormone concentrations in non-malignant breast tissue samples from postmenopausal human subjects normalized by sample..................69

Table 4.1. Final amounts (fmol/mg) of exogenous E2 standards spiked into eleven breast tissue samples obtained from lactating rats.................................................................79

Table 4.2. Final amounts (fmol/mg) of exogenous E2 standards spiked into two sets of breast tissue samples obtained from lactating rats.................................................................80

Table 4.3. Estradiol values measured using the integrated DMF method with on-chip immunoassay in core needle biopsy (CNB) tissue samples collected from fifteen postmenopausal subjects before and after treatment with AIT.................................................................89
Table 5.1. Final concentrations (ng/mL) of exogenous standards spiked into five endometrial tissue sub-samples

Table 5.2. HPLC-MS/MS conditions and multiple reaction ion monitoring (MRM) transitions for eighteen light and heavy target analytes

Table 5.3. Calibration data for nine target analytes

Table 5.4. Measured analyte amounts in endometrial tissue biopsy samples from human subjects normalized by sample mass
List of Figures

Figure 2.1. Sensitivity improvement for measurement of estradiol by derivatization with dansyl chloride........................................................................................................................................33

Figure 2.2. HPLC-MS/MS calibration plots for derivatized estradiol, androstenedione, testosterone, and progesterone obtained in week 1.......................................................................................................................................35

Figure 2.3. Digital microfluidic extraction/cleanup from tissue samples.................................................................................................................................37

Figure 2.4. Sample cleanup.................................................................................................................................................................................................38

Figure 2.5. Extraction time ...................................................................................................................................................................................................39

Figure 2.6. Extraction efficiency.......................................................................................................................................................................................44

Figure 3.1. Tissue and serum estradiol levels in seven premenopausal women prior to and following bilateral salpingo-oophorectomy....................................................................................................................................59

Figure 3.2. Tissue and serum estradiol levels in five postmenopausal women prior to and following bilateral salpingo-oophorectomy........................................................................................................................................60

Figure 3.3. Tissue androstendione levels in seven premenopausal women prior to and following bilateral salpingo-oophorectomy........................................................................................................................................61

Figure 3.4. Tissue androstendione levels in five postmenopausal women prior to and following bilateral salpingo-oophorectomy........................................................................................................................................62

Figure 3.5. Tissue testosterone levels in five premenopausal women prior to and following bilateral salpingo-oophorectomy........................................................................................................................................63

Figure 3.6. Tissue testosterone levels in five postmenopausal women prior to and following bilateral salpingo-oophorectomy........................................................................................................................................64

Figure 3.7. Tissue and serum progesterone levels in seven premenopausal women prior to and following bilateral salpingo-oophorectomy....................................................................................................................................65
Figure 3.8. Tissue and serum progesterone levels in postmenopausal women prior to and following bilateral salpingo-oophorectomy. ............................ ................................................................. 66

Figure 4.1. A personalized regime for aromatase inhibitor therapy (AIT) ................................................................................................. 73

Figure 4.2. Digital microfluidic (DMF) extraction of estradiol (E2) from core needle biopsy (CNB) samples .............................................................................................................. 83

Figure 4.3. Digital microfluidic (DMF) quantification of E2 by on-chip immunoassay ................................................................................................................................. 85

Figure 4.4. Comparison between on-chip DMF immunoassay and off-chip HPLC-MS/MS ................................................................................................. 86

Figure 4.5. Rat breast tissue samples extracted and analyzed using the integrated DMF immunoassay technique ................................................................................................. 88

Figure 5.1. Consistency of analyte concentration in endometrial tissue ........................................................................................................ 102

Figure 5.2. Quantification of endogenous NE, EP, PG, HC and E2 in human endometrial tissue samples by standard addition ........................................................................................................ 104
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-HG</td>
<td>2-Hydroxyglutarate</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>µTAS</td>
<td>Micro Total Analysis System</td>
</tr>
<tr>
<td>AD</td>
<td>Androstendione</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AG</td>
<td>Antigen</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under The Curve</td>
</tr>
<tr>
<td>AIT</td>
<td>Aromatase Inhibitor Therapy</td>
</tr>
<tr>
<td>DDA</td>
<td>Data Dependent Acquisition</td>
</tr>
<tr>
<td>DIA</td>
<td>Data-Independent Acquisition</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DESI</td>
<td>Desorption Electrospray Ionization</td>
</tr>
<tr>
<td>DMF</td>
<td>Digital Microfluidics</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>ET</td>
<td>Embryo transfer</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked-Immunosorbent Assay</td>
</tr>
<tr>
<td>EP</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>E2</td>
<td>β-Estradiol</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin Fixed Paraffin Wax Embedded</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier Transform</td>
</tr>
<tr>
<td>GP</td>
<td>Glycerophospholipid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin And Eosin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HT</td>
<td>Hormone Therapy</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>HC</td>
<td>Hydrocortisol</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ISH</td>
<td>In Situ Hybridization</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
</tr>
<tr>
<td>ITO</td>
<td>Indium Tin Oxide</td>
</tr>
<tr>
<td>IS</td>
<td>Internal Standard</td>
</tr>
<tr>
<td>LOC</td>
<td>Lab On A Chip</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit Of Quantification</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit Of Detection</td>
</tr>
<tr>
<td>LLE</td>
<td>Liquid Liquid Extraction</td>
</tr>
<tr>
<td>MEOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-Assisted Laser Desorption/Ionization</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple Reaction Monitoring</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-Charge Ratio</td>
</tr>
<tr>
<td>NE</td>
<td>Nor-Epinephrine</td>
</tr>
<tr>
<td>PME</td>
<td>Peptide Mass Fingerprinting</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier Tube</td>
</tr>
<tr>
<td>PPMs</td>
<td>Porous Polymer Monoliths</td>
</tr>
<tr>
<td>PCT</td>
<td>Pressure Cycling Technology</td>
</tr>
<tr>
<td>PG</td>
<td>Progesterone</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SRM</td>
<td>Selected Reaction Monitoring</td>
</tr>
<tr>
<td>SERMs</td>
<td>Selective Estrogen Receptor Modulators</td>
</tr>
<tr>
<td>SWATH</td>
<td>Sequential Window Acquisition Of All Theoretical Fragment</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex Hormone Binding Globulin</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Denaturing Gel Electrophoresis</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>TS</td>
<td>Testosterone</td>
</tr>
<tr>
<td>TOF</td>
<td>Time Of Flight</td>
</tr>
<tr>
<td>TMAs</td>
<td>Tissue Microarrays</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor, Nodes, Metastasis</td>
</tr>
<tr>
<td>T3</td>
<td>Triiodothyronine</td>
</tr>
<tr>
<td>T4</td>
<td>L-Thyroxine</td>
</tr>
<tr>
<td>TNFC</td>
<td>University Of Toronto Nanofabrication Centre</td>
</tr>
</tbody>
</table>
Chapter 1
Review of Tissue Biomarker Quantification: Methods and Applications

This dissertation describes the development of a suite of new tools that are useful for analyzing tissue samples for biomarkers of disease. This work required me to make contributions in the areas of tissue biomarker quantification, tissue sample processing, tissue analysis techniques, and microfluidics. Each of these topics is briefly reviewed below.

1.1 Tissue Biomarker Quantification Applications

Scientists have long sought to identify ways to detect and quantify biomarkers for early stage diagnostics to enable selection of optimal therapeutics and/or medical procedures that are most suitable for each individual case. Many biomarker assessment tests depend on the information obtained from small sections of tissue samples that are used to stage and diagnose a specific disease. Key topics in this area include histopathology, proteomics and metabolomics, microarrays, molecular assays, and mass spectrometry-based imaging. Advantages and disadvantages for each of these topics are described below.

1.1.1. Tissue Histopathological Applications

For many decades, histopathological examination has been considered the gold standard in diagnostics of tissue-specific diseases. Immunohistochemistry (IHC) is a technique used to determine tissue distribution of an antigen which in return reflects a healthy or diseased status. In IHC a tissue sample is removed from the subject by surgical or needle-biopsy and then is frozen or chemically preserved in a fixative (see section 1.2.1) such as formaldehyde (which helps maintain cell structure), after which multiple steps (including some or all of dehydration, embedding in wax, sectioning with a microtome and antigen retrieval) are performed. Antibody staining is then used to probe the tissue for its target antigen. Staining antibodies are either directly labeled with fluorescent dye, enzymes, or a radioactive element or are allowed to further react with a secondary antibody bearing a
label. The overall process results in a stained tissue section that can be visualized using a microscope and/or with the aid of image processing software.\textsuperscript{2,3} IHC enables localization of cellular components without compromising the integrity of cellular architecture which makes it suitable for detection of tumor specific antigens such as prostate specific antigen (PSA) in prostate cancer;\textsuperscript{4} detection of cell proliferation markers such as phosphohistone H3/MART1, Ki67/MART1 in primary cutaneous melanoma;\textsuperscript{5} detection of oncogenes and tumor suppressor genes;\textsuperscript{6} and in the measurement tumor microvessel density as an indication to development of metastasis.\textsuperscript{7}

IHC has also been used for the detection of cancer of unknown primary site (CUP); for example, the 533-amino acid protein, prostein (P501S) that is expressed only in prostate tissue, has been used as a biomarker to demonstrate prostatic origin of metastatic tumors of unknown primary site.\textsuperscript{8} Likewise, differential expression of cytokeratins (CK) at primary and metastatic tumor sites have been used to distinguish primary ovarian carcinoma and colorectal adenocarcinoma that are metastatic from the ovary.\textsuperscript{9} Micrometastases can also be detected by IHC, by targeting epithelial differentiation antigens of individual micrometastatic carcinoma cells in secondary organs.\textsuperscript{10}

Another form of histopathological assessment is done by staining tissue sections using haematoxylin and eosin (H&E). This technique is employed to examine tissue samples to ‘stage’ (i.e. determine the degree of spreading) and 'grade' (i.e. determine the aggressiveness) tumors. H&E staining helps highlight the underlying tissue structures. Hematoxylin (a basic dye) reacts with acidic or basophilic cell components including DNA containing nuclei, RNA containing ribosomes and the endoplasmic reticulum producing a purplish blue color. While eosin (an acidic dye) reacts with acidophilic structures including cell wall and the cytoplasm producing a reddish or pink color. Color changes can be used to verify changes in cell and tissue phenotypes with the aid of microscopy.\textsuperscript{11} For example, light microscopy offers a resolution of features with dimensions as small as 200-250 nm, higher resolution images can be generated by electron microscopy if necessary, which can resolve features with much smaller dimensions (i.e., ~0.2 nm).
In histopathological tissue assessment, a stepwise diagnostic approach is followed, resulting in a numerical score for specific stages and grades.\textsuperscript{12,13}

Histopathological tissue grading is done by quantitatively measuring characteristics resulting from changes in cell phenotype such as: DNA ploidy; nuclear morphometry (nuclear size, shape and clustering); densitometric characteristics (nuclear density and chromatin disorganization); and cell counts by measuring mitotic indices.\textsuperscript{3,14}

Histopathological staging of tumors follows a three component “TNM” (tumor, nodes, metastasis) system. Numerical values are assigned to the three aspects of the tumor based on pathological assessment, where primary tumor (size, the degree of spread into surrounding tissue), lymph nodes (number and site) and finally metastasis (present or absent) are all assessed and each component is given a score. Combining all the three scores of the three components can lead to final assignment of the tumor stage.

In histopathological applications, formalin fixed paraffin wax embedded (FFPE) tissue samples are typically used. FFPE processing requires many hours of laborious work (e.g., the time between formalin fixation to sectioning ranges from 36-48 h); this challenge can be partly alleviated by using automated sample preparation instruments, which are used to constantly agitate the samples and allow for multiplexed sample processing. Using such systems can reduce the time required for sample analysis by at least 24 h.\textsuperscript{15}

Automated tissue staining platforms are also available; some challenges to such platforms arise from their restricted capabilities allowing for a limited number of reagent, antibodies and protocols to be utilized.\textsuperscript{16}

In some clinical settings, time is crucial (for example, an intra-operative histopathological report may be required within minutes to help with in-surgery decision making\textsuperscript{17}); in such cases, freshly frozen tissue samples are used. Freshly frozen tissue can be stained and processed within minutes, however the quality of the information obtained by such method is inferior to FFPE tissue sections and may compromise the interpretation of the results.\textsuperscript{18}

Standard scoring methods employed in histopathology follow broad guidelines that depend on visual interpretation by a qualified practitioner.\textsuperscript{19} This inherent subjectivity
leads to inconsistency in diagnoses, which further results in poor reproducibility in image interpretation. Computer-aided digital image analysis has been introduced as an alternative to standard techniques in an effort to minimize human bias, towards a more objective decision making in pathology.\textsuperscript{16,20} But the shortcomings of current practice in histopathology suggest an important role for rapid quantification of validated biomarkers; this has been a driving motivation in the work described in this dissertation.

1.1.2. Tissue Proteomics and Metabolomics

There is considerable interest in the development of innovative protocols and methodologies that allow for proteomic and metabolomic profiling of tissue samples. These methods could serve as information-rich tools for disease diagnostics.\textsuperscript{21} Several approaches have been reported for proteomic and metabolomic analysis of tissue samples, here I highlight some of the most frequently reported techniques.

1.1.2.1 Tissue metabolomics

Tissue metabolomics aims to identify and quantify small-molecule ($<1500$ Da) metabolites in tissue samples.\textsuperscript{22} These studies can be classified into three main categories: (1) targeted metabolomics, in which one or a few target compounds are quantified in absolute manner using external standard calibration plots (much of the work described in this thesis fits into this category), (2) metabolite profiling, which focuses on the analysis of analytes related to a specific class of compounds or a biochemical pathway and (3) global metabolomics, which aims to generate a comprehensive unbiased quantitative and qualitative analysis of all classes of metabolites in a given sample with no restrictions on the physical and chemical properties of the detected analytes. For targeted metabolomics and metabolite profiling, a single analytical platform is usually sufficient for analyte detection; however, in global metabolomics a combination of analytical techniques is usually employed in an attempt to allow for comprehensive analysis of compounds of diverse physico-chemical properties.\textsuperscript{23}

A variety of analytical techniques have been reported for metabolomic analysis of tissue samples. The most commonly employed analytical platforms depend on nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), which is usually coupled to
a separation technique such as high performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE).

NMR spectroscopy is a non-destructive analytical technique capable of providing chemical structure information by probing the chemical environment of nuclei with non-zero spin – e.g., $^1\text{H}$, $^{13}\text{C}$, $^{31}\text{P}$ and $^{19}\text{F}$. NMR is a robust and highly reproducible technique, that is well suited for metabolite fingerprinting via its capability to simultaneously detect a wide variety of chemically diverse metabolites. A typical NMR metabolomic workflow includes sample preparation, recording NMR spectra according to a standardized protocol, and using statistical analysis of the collected data to identify metabolite differences between different test groups (e.g., diseased versus healthy group). Tissue preparation for NMR involves a number of general steps (see section 1.2.1) followed by stepwise addition of solvents for the extraction of target analytes. NMR have been successfully employed to identify metabolic biomarkers of sepsis in rat lung tissue samples, and to detect cancer metabolite biomarkers in brain, breast and prostate tissue. In some NMR tissue metabolomic applications, specialty techniques such as magic angle spinning (MAS) NMR spectroscopy can be used to identify analytes in intact tissue samples without prior treatment.

A key limitation to NMR is its inherent low sensitivity, which makes it suitable only for detection of metabolites present at medium to high abundance with picomole limit of detection or higher. Recently, a digital microfluidic platform capable of interfacing droplets of analyte with NMR microcoils in a high-field NMR spectrometer was reported as a solution to this limitation. The work described in this thesis does not employ NMR for analyte detection; however, with this recent advancement, sensitive analysis of biomarkers in tissue samples by means of DMF-NMR could be made possible in the near future.

Another general approach to tissue metabolomics is based on mass spectrometry, which offers great potential for the identification of metabolites, exquisite capabilities for quantitative analysis, high concentration-sensitivity and comparatively a large dynamic range. Typically a separation technique (e.g., HPLC, GC or CE) is interfaced to a MS
detector to reduce sample complexity, separate isomeric and isobaric metabolome components, and to help identify chemical structure of target analytes.

HPLC is the separation technique that is most frequently hyphenated to MS detection for metabolomics studies. HPLC is particularly well suited for the analysis of labile and non-volatile compounds, and was the method used in many of the methods described in this dissertation. HPLC can be implemented in various modes, the most common being reversed-phase (RP) chromatography. In this mode, a hydrophobic stationary phase materials (e.g., C8 or C18 bonded phases) is used to separate analytes on the basis of polarity. Another common mode is hydrophilic interaction liquid chromatography (HILIC), which employs neutral, polar or ionic stationary phase material (e.g., silanol bonded phases, amino or anionic bonded phases) to separate neutral, hydrophilic, polar and ionic compounds.  

Although different modes of HPLC can be used to separate several classes of analytes, finding a single stationary phase suitable for many different constituents in a metabolome with diverse physicochemical properties can be a challenging task.

Capillary electrophoresis (CE) is an alternative to HPLC for separations of metabolites upstream of mass spectrometry. CE has many advantages, including high separation efficiency, low sample volume requirement, and low-cost operation. While the small sample volume is useful for some applications, it can limit others, as small volumes may not contain appreciable numbers of analytes present at very low concentrations. Online sample preconcentration steps such as sample stacking, transient isotachophoresis, and sweeping can be used to overcome this limitation; however, a single online sample preconcentration protocol might not be sufficient for the separation of many different classes of analytes, which may require multiple on-line focusing techniques to be performed for better analyte coverage.

Gas chromatography (GC) is a third separation technique that is often coupled to mass spectrometry for metabolomic analysis. GC offers high resolution and good sensitivity for analytes present at low abundance; however, chemical derivatization steps are often required to improve volatility or chemical stability, which prolongs sample preparation time. Inefficient sample derivatization may also complicate interpretation of results.
Tissue sample preparation for mass spectrometry-based metabolomics studies involves many steps (described in details in section 1.2). For example, careful attention must be paid to tissue handling in order to minimize variation caused by degradation or formation of metabolites as a result of enzymatic activity. Techniques such as freezing in liquid nitrogen and quenching in organic solvents have been used to limit this effect.\textsuperscript{34}

Tissue metabolomics analyses have been routinely performed on larger sections of tissue samples (>100 mg); however there have been recent attempts to utilize smaller tissue biopsies for metabolomic research. Huan et al.\textsuperscript{35} describe a method for metabolomic profiling of tissue biopsies (~5 mg) based on methanolic fixation/extraction of metabolites, followed by chemical isotope labeling (CIL) liquid chromatography mass spectrometry (LC-MS). The method is novel in terms of its ability to perform comprehensive metabolite coverage from small sections of prostate tissue samples; some limitations to this technique include prolonged tissue solvent incubation (~ 2 h), and the need to perform several manual sample processing step e.g. mixing, splitting and labeling. The work presented in this thesis describes an attractive solution to this problem in which small tissue biopsies can be actively mixed with organic solvents (which can lead to minimization of solvent incubation time); in addition, DMF allows for precise sample processing steps to be performed e.g. splitting, mixing and merging all in an automated fashion.\textsuperscript{36}

1.1.2.2. Tissue Proteomics

Numerous approaches have been reported for proteomic tissue processing including affinity based protocols and mass spectrometry based techniques.

Affinity based proteomics protocols depend on the production of antibodies highly specific for target antigens. Once generated, antibodies are utilized in an array of methods for targeted protein detection and/or quantification in complex tissue samples. Different version of affinity-based proteomics have been utilized (e.g., immunoblotting, enzyme-linked immunosorbent assay or ELISA, and microarray based techniques) for different applications, depending on the type of affinity reagent and the detection system being used. Immunoblotting and ELISA both provide means to quantify protein in tissue lysates
based on specific antigen-antibody interaction; the main difference between the two techniques is the initial protein separation steps that are employed in immunoblotting but not in ELISAs.

In immunoblotting tissue proteomics, proteins are first extracted from clinical samples, fractionated according to their size using sodium dodecyl sulfate polyacrylamide denaturing gel electrophoresis (SDS-PAGE), and immobilized onto a membrane support. The membranes are then blocked to reduce nonspecific binding, exposed to antibodies against target antigens, and finally the antibody-antigen reactions are identified by colorimetric, chemiluminescent, radioactive or fluorescence-based detection. Changes in protein expression profiles have been monitored in human stomach xenografts, and in gastric adenocarcinoma tissue samples employing immunoblotting in an attempt to study cell dynamics that reflect healthy or diseased status. Tissue sample preparation for immunoblotting is important for accurate analysis; cell extracts are usually processed on ice and/or protease inhibitors are included in the lysis buffer to reduce the effects of proteolytic enzymes. Finally, the composition of lysing buffer (detergent type, salt concentration, pH and the presence or absence of chelating agents) is carefully controlled to preserve analyte solubility.

Affinity based proteomics have many advantages, including the capacity to exploit automated systems for high-throughput analysis, high assay-sensitivity (i.e., these techniques can detect low abundance protein with zeptomole limits of detection), but they also suffer from disadvantages such as cross-reactivity, the challenging nature of affinity reagent development, matrix interference, limited capabilities for multiplexed detection and inter-laboratory variation.

Another general approach to tissue proteomic analysis depends on mass spectrometry (MS). In fact, for some applications, MS-based techniques have recently emerged as the gold standard for tissue proteomic analysis, because of to the technique's ability to cover thousands of analytes in a complex sample matrix. Depending on the application, a number of mass spectrometry-based techniques have been used for tissue-sample analysis (which has been extensively reviewed in previous reports). Here I highlight some of the most frequently reported techniques.
A key strategy used for protein identification by MS has been to separate proteins in the sample by gel electrophoresis (one dimensional or two dimensional). In this technique, spots of separated protein on the gel are excised, digested into peptides using a sequence specific protease enzyme e.g. trypsin and analysed by MS. Gel separated proteins have been utilized for protein identification by peptide mass fingerprinting (PMF) and by peptide sequencing, employing matrix-assisted laser desorption/ionization (MALDI) Time of Flight (TOF) or Electrospray Ionization (ESI) Time of Flight (TOF) for the former and ESI-MS/MS for the latter.\textsuperscript{44,46,47}

Another strategy for protein identification by MS depends on high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Using liquid chromatography allows for analysis of complex mixtures without the need for prolonged sample-preparation steps.\textsuperscript{43} One of three main approaches is typically followed: (1) targeted analysis\textsuperscript{48} focusing on a subset of protein analytes of interest, (2) data dependent acquisition (DDA)\textsuperscript{43,49} and (3) data-independent acquisition (DIA).\textsuperscript{43,49,50} Each of these techniques has become increasingly powerful as tandem mass spectrometry (MS/MS) data processing methods have improved. For (1) absolute quantification (with isotope-labeled standards) is popular; much of the work described in this thesis fits into this category with focus on small molecule analysis. For (2-3), absolute quantification remains a challenge, but the capacity to identify all (or a large fraction) of analytes in a particular sample has emerged as a powerful tool for discovery and analysis.

Tissue proteomics analyses are routinely performed on larger sections of tissue samples (>100 mg), and they often involve multiple time-consuming, labor-intensive processing steps including tissue lysis, homogenization and enzymatic digestion. Smaller tissue samples are (for obvious reasons) better for the patient, and there have been recent attempts to scale the size down. For example, a recent report\textsuperscript{51} utilized formalin fixed paraffin wax embedded tissue biopsies (~1 mg) for protein quantification; in this report, tissue lysis, homogenization and digestion steps were all done under pressure cycling technology (PCT), a technique that employs alternating cycles of hydrostatic pressure ranging from ambient up to ultra-high levels (~ 100,000 psi) to break down cells in the sample with subsequent release of cellular proteins. After lysis and digestion, cleanup
steps on C18 solid-phase extraction cartridges were done, and the peptide samples were further analyzed using sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS). Even with this new technique, prolonged tissue processing time was required (~ 6-8 h), on top of the time required for tissue chemical fixation (12-48 h).

1.1.3. Tissue Microarrays

Tissue microarrays (TMAs) represent another tool that is used to quantify biomarkers in tissue samples. In TMAs, multiple samples are assembled on a single histological slide allowing for high throughput analysis. To prepare TMAs, small cylindrical tissue cores are extracted from regions of interest in paraffin-embedded tissue samples; these cores are placed with precise spacing in a new paraffin block creating a tissue array of hundreds of samples for analysis. Recipient blocks are further sliced and sectioned into microarray slides (4-8 µm in thickness) containing small discs of tissue specimens (0.6 mm in diameter). Tissue microarray slides can be used for histolopathology, immunohistochemistry, (as discussed above) or for molecular genetic testing of DNA and RNA. So far, applications of this technique have been limited to molecular screening/analysis. Some critical limitations of this technique arise from its inability to individually address different samples with different sample processing regimens unless performed manually, which is time consuming and counters the purpose of having hundreds of samples assembled on one slide. TMA technology also lacks the presence of standardized protocols for data collection and validation. The work described in this thesis describes techniques that might eventually be used for multiplexed analysis of tissue cores, combined with the flexibility to treat each core differently (e.g., different procedures for different analyses).

1.1.4. Tissue Molecular Testing

There are a number of methods used for nucleic acid analysis in tissue samples. In situ hybridization (ISH) is one such technique, which can provide invaluable insights to disease pathogenesis. In ISH, tissue sections are exposed to labeled complementary probes to messenger RNA (mRNA) analytes of interest; subsequent analysis by microscopy reveals the location of the analytes relative to the structure of the sample.
Because mRNA is the target of the assay, removal of proteins and inactivation of nucleases are important steps in sample preparation (e.g., proteinase K is commonly used for this purpose\textsuperscript{56}).

ISH has been mainly performed on formalin-fixed paraffin wax-embedded tissue samples. The main problem with this type of sample is that the high temperature embedding process may lead to degradation of mRNA, and the fixation process may hinder probe access to target tissue mRNA. Freshly frozen tissue samples on the other hand do not present this problem; however, frozen samples exhibit poorly preserved tissue architecture which hinders the collection of information on the spatial distribution of mRNA at the tissue. Given these issues, tissue treatment is critical in ISH experimental design, to optimize and enhance probe access to target mRNA on different types of tissue sections.\textsuperscript{57} In recognition of this challenge, many different strategies have been explored; for example, microwave heating has been introduced as a technique to deactivate biogenic enzymes in formalin fixed and frozen tissue sections.\textsuperscript{57,58} Further, microwave treatment was also found to be useful in enhancing the action of proteinase-K in formalin-fixed tissue sections, and additionally assisted in denaturing the target mRNA to enable better probe access.\textsuperscript{57}

A variety of labeled probes have been utilized for ISH including the commonly used ribonucleic acid (RNA)- or deoxyribonucleic acid (DNA)-type probes. Commonly, the probes are coupled with fluorescent-, radio- or antigen labels, which can be detected with fluorescence microscopy, autoradiography and immunohistochemistry detection systems, respectively.\textsuperscript{55,59-61} In the work described in this thesis, non-nucleic acid analytes were evaluated exclusively by mass spectrometry and immunoaffinity techniques (and no attempts at “imaging” were made). But some of the processes described in my work (with appropriate modifications) might be useful for ISH and related techniques in future work.

Another current focus of molecular assays for tissue sample analysis is based on gene expression analysis using DNA microarrays. In this technique, RNA analytes are extracted from a sample and then exposed to an array of complementary probes on a surface. These systems can be used for a wide range of applications including disease diagnostics, drug discovery and gene discovery research.\textsuperscript{62-64}
A typical DNA microarray consists of a miniature silicon chip or a glass slide containing up to 20,000 rows of dots of oligonucleotide strands (which can be synthesized in situ) or complementary DNAs (cDNAs) that are complementary to RNA transcripts of interest. DNA microarrays have been used to evaluate gene expression in both fixed and freshly frozen tissue samples. Non-formalin-fixed samples have shown better performance over formalin-fixed ones, since they do not compromise tissue RNA and protein status. DNA arrays are designed based on the application for which it is going to be used, e.g. tumor tissue classification; target tumor suppressor or gene identification; tumor invasion; drug discovery; and tumor biomarkers. A typical gene expression analysis experiment involves the extraction of RNA from tissue samples followed by its amplification and labelling (most commonly used labels are fluorescence based). After a washing step, labeled RNA is allowed to hybridize the array in presence of a reference RNA (labeled with a different fluorescent dye). Detection and quantification of the hybridized target is done by measuring fluorescence intensity to determine relative abundance of nucleic acid sequences in the target.

1.1.5. Mass Spectrometry-Based Imaging Applications
Imaging mass spectrometry has been introduced as a powerful tool for tissue biomarker discovery that obviates the need for extensive tissue pretreatment. Different mass spectrometry ionization techniques have been employed for this purpose—mainly matrix-assisted laser desorption/ionization (MALDI) and desorption electrospray ionization (DESI). In MALDI, tissue samples are first mixed with MALDI matrix (to allow for co-crystallization of matrix and analytes). This can be implemented by spraying the matrix over the sample and allowing it to dry, by sublimating the matrix and condensing it to the tissue surface, or by means of automated tissue spotters. After application of matrix, short UV laser pulses are used to desorb and ionize analytes from specific locations within the sample into the gas phase. The molecular weights of the ions are then determined by mass spectrometry (see section 1.3.1), typically with a time-of-flight (TOF) mass analyzer. An ion image can then be generated by plotting the intensity of a given ion on a coordinate system representing the relative location of that ion in the tissue sample, creating a visual representation of the molecular information. These
images can be further used to compare the information from different locations of the tissue e.g. cancerous versus normal tissue.\textsuperscript{71}

MALDI-imaging has focused on identification and relative quantification of different classes of compounds such as proteins and endogenous peptides (especially those with masses less than 15 kDa\textsuperscript{72}), lipids,\textsuperscript{73} and drugs and metabolites.\textsuperscript{74} For protein analysis, these studies typically make use of formalin-fixed tissue sections that have been heat-treated followed by in-situ tryptic digestion.\textsuperscript{75} Alternatively, non-formalin based tissue fixation can be used to retrieve proteins without the need for tryptic digestion.\textsuperscript{76} The resolution of MALDI-imaging techniques is determined by the laser beam diameter (20-200 µm on average with some advanced instruments reaching a laser beam focusing down to 1 µm\textsuperscript{77}), and the spacing distance between the different ablation spots.\textsuperscript{68}

MALDI imaging offers several advantages, from high resolution mapping to extensive coverage of several classes of analytes; nevertheless the quality of the data obtained by MALDI imaging is highly dependent on the quality of matrix coating (uneven matrix distribution compromises reproducibility and repeatability) and its components. Further, MALDI is limited to analyzing analytes with large masses, as matrix-related peaks crowd the low-mass region of the spectrum.\textsuperscript{78}

Like MALDI, DESI\textsuperscript{79} is a soft ambient ionization technique that is useful for evaluating ions that fragment when subjected to traditional ionization modalities. But unlike MALDI, DESI does not exhibit the mass range limitation of analytes to be ionized. In DESI, charged species are created from an electrospray emitter and are brought to the sample surface. The charged species collide with sample surface with assistance from high pressure sheath gas, leading to the formation of secondary ions which are subsequently transferred to a mass analyzer.\textsuperscript{79} Images are generated by rastering the ion emitter relative to the sample, generating ion maps similar to the case for MALDI imaging (described above). The signal intensity of specific classes of analytes can be enhanced by controlling different operation parameters – e.g., electrospray solvent polarity, salt concentration and pH, source pressure and applied voltages.\textsuperscript{78} Coupling DESI to mass analyzers with higher resolution capabilities can also be used to increase analyte selectivity and reduce problems encountered due to sample complexity.\textsuperscript{80} Several
applications have been reported for tissue imaging by DESI including metabolite profiling in breast cancer tissue samples, and lipid distribution in normal and cancerous tissue. DESI-imaging has been also used to differentiate tissue samples as cancerous or normal based on the absence or presence of fatty acid or glycerophospholipid (GP) markers.

Rapid evaporative ionization mass spectrometry (REIMS) has recently emerged as a tool that allows characterization of the aerosol produced during electrosurgical tissue dissection procedures. This technology which is referred to as the intelligent knife (iKnife) is used to determine tissue type according to resulting mass spectrometric profiles. iKnife allows real-time-characterization and requires no sample preparation.

Mass spectrometry-based tissue imaging has become an important new tool in the analyst’s toolbox. But the applications for this technique are quite different from those described in this dissertation. Specifically, the work introduced in this dissertation is focused on quantification of analytes (without preserving “images” or structural information), while imaging techniques provide structural information, but are at best semi-quantitative.

1.2. Tissue Processing Procedures

The processing steps applied to tissue samples prior to analysis are critical. Most samples must be subjected to some form of initial handling steps, and those that are not being imaged are typically subjected to an extraction process. Initial steps and sample extractions are reviewed below.

1.2.1. Initial Tissue Handling Steps

Depending on the application for which a tissue sample is going to be used, different initial tissue handling steps may be required. For applications requiring preservation of tissue morphological characteristics e.g. tissue imaging applications, tissue stabilization by fixation is usually employed. However, other applications focus on protecting target analytes from degradation and maximizing target-analyte recovery, e.g. tissue
metabolomics applications, in such applications initial tissue stabilization by freezing followed by homogenization/disruption procedures are usually utilized. Stabilization measures are required to prevent biological tissue from decaying and to maintain its structural integrity. The most common method is to apply a chemical fixative agent to the sample, with the ultimate goal of preventing tissue cells from degradation and maintaining cellular and morphological structure. A chemical fixative can work by different mechanisms including dehydration and cross linking. The most widely used fixative is 10% neutral buffered formalin (~4% formaldehyde in phosphate buffer saline), which is typically applied at 4°C. This agent works by cross-linking amino groups in tissue proteins through the formation of methylene bridges (-CH2-), which is sufficient to preserve a wide range of tissue components. The duration of fixation process has been reported to be around 1 h for each 1 mm of tissue thickness; in practice, typical tissue fixation time ranges between 12–48 h. Reports have shown that subjecting the tissue to formalin for longer than 24 h can result in degradation of DNA, while shorter fixation durations (3-6 h) and low-temperature formalin fixation have been associated with higher yield of high-molecular weight DNA. Formalin fixed tissue samples have also been reported to show poor performance in some applications in which RNA is the analyte, as some RNAses are apparently not inhibited by the mixture. There are many variations that have been used with formalin fixation protocols. For example, samples are sometimes exposed to microwave treatment of tissues for ~2 min to raise the temperature to ~60 °C post formalin fixation, which has been hypothesized to reduce enzymatic degradation of nucleic acids and to also cause mRNA denaturation enabling better access to the tissue embedded molecules. Other fixation solvents and techniques have also been used, including organic solvents and alcohol-based fixatives such as gluteraldehyde, ethanol, methanol and acetone.

Following fixation, tissue samples are typically treated by a number of steps to convert the sample into a state that can be stored for long periods—these steps include dehydration, clearing, wax infiltration and embedding. Dehydration is carried out by exposing the sample to an alcohol to remove water, enabling wax infiltration into the tissue. A dehydration agent is typically applied in series with increasing concentrations to
ensure gradual replacement of water in the tissue to avoid excessive disruption. After dehydration, a hydrophobic clearing agent (typically a hydrocarbon solvent) such as xylene or toluene is used to remove the alcohol, rendering the tissue compatible with wax infiltration. Paraffin wax has been the most popular agent for infiltration; wax mixtures (including purified paraffin wax and additives resins such as styrene or polyethylene) are typically applied to the tissue at 60°C and allowed to infiltrate through it, before cooling to 20°C allowing it to solidify, after thorough wax infiltration, the infiltrated tissue is transferred into a mould filled with molten wax and placed into it for final wax embedding to enable tissue sectioning at a later stage by microtome.  

Tissue stabilization by fixation and subsequent processing is a long process that requires prolonged hours of laborious work, 12-48 h for fixation and 6-10 h for the rest of the process. In the work described in this dissertation, fixation was not used, primarily because preservation of sample-structure was not required for the applications that motivated the work. But given the existence of tissue-banks bearing millions of formalin-fixed, paraffin-infiltrated samples correlated with known patient histories and outcomes, I propose that in the future, it might be interesting to develop digital microfluidic methods to process and analyze these types of samples.

Another protocol commonly used for preserving tissue samples from degradation is through freezing. In this technique, tissue samples are instantly frozen by exposure to a suitable medium such as dry ice or by immersing in liquid nitrogen. Frozen tissue can be stored at -80°C until further use; recent reports have shown that tissue stored at -132°C or lower exhibited better long-term viability and less enzyme degradation.

Tissue stabilization by freezing is much less tedious than fixation. But a key drawback of freezing is that careful attention must be paid to handling the sample after thawing (prior to analysis) to avoid degradation of the preserved structure for imaging. Imaging was not a goal for the work described in this dissertation; thus, instant freezing was the method used here.

For applications that require imaging, it is important to preserve the structure of the sample. For other applications, however, the structure of the sample is unimportant; in
these cases, it is important to extract as much analyte as possible for subsequent analysis. For such applications (including all of the new methods described in this thesis), tissue homogenization/disruption is a critical early step in sample processing.

Chemical and mechanical disruption methods are used for homogenization. Chemical disruption is typically applied to samples of cells, and involves exposure to lysis buffers/solutions such as detergents/surfactants that can disrupt and solubilize cell membranes and assist in protein denaturation\(^\text{91,92}\) or chaotropic agents that can disrupt weak inter-molecular interactions like hydrogen bonding and hydrophobic interactions,\(^\text{93}\) and enzymes that can degrade cell wall or structural proteins (often used in DNA extraction applications\(^\text{94}\)). An alternate form of disruption, applied most often to tissue samples, is achieved by application of mechanical or physical forces. These techniques can take different forms, including shearing forces through a blender, grinding forces with mortar and pestle, beating forces applied through direct impact of the tissue cells with a ball or hammer, and shocking forces by applying acoustic energy to the sample.\(^\text{95}\) Depending on the objective of the experiment and the target compound or class of compounds to be analyzed, one homogenization method or a combination of several maybe employed to attain the required end-results.

1.2.2. Sample Extraction

For non-imaging applications, extraction is an intermediate step between sample collection and analysis that is used to simplify the sample matrix (to prevent unwanted interferences from matrix components) and to increase the sensitivity of the detection method towards the target analyte.

1.2.2.1. Liquid-Liquid Extraction

A common form of sample clean-up (from solution) is liquid-liquid extraction (LLE). This process depends on the differential distribution of sample components in two immiscible liquid phases; in practice one of the two phases is aqueous while the second phase is organic.\(^\text{96}\) Favorable solubility of the target analyte in one of the two phases allows for it to be readily extracted into that phase, leaving behind unwanted matrix components in the other phase. In the case of an aqueous/organic two phase system, polar
low-molecular weight substances are extracted into the aqueous phase, while less polar water-insoluble substances are extracted into the organic phase, governed by the distribution coefficient (K) of each analyte. The distribution coefficient acts as a quantitative measure of the distribution of a given analyte between two immiscible liquid phases so that at equilibrium the ratio of its concentration in each phase is equal to the ratio of its solubility in each (equation 1).

\[
K = \frac{S_1}{S_2} = \frac{\frac{p}{V_A}}{\frac{a-p}{V_B}} \quad (1)
\]

Where:

- \(K\) = distribution coefficient.
- \(S_1\) = solubility of analyte in solvent A.
- \(S_2\) = solubility of analyte in solvent B.
- \(a\) = mass of the analyte in the solution before extraction.
- \(p\) = mass of the analyte in \(V_A\) volume of solvent A after extraction.
- \(a-p\) = mass of the analyte in \(V_B\) volume of Solvent B after extraction.

In a LLE, once equilibrium is reached, the analyte and matrix molecules will be distributed in the solvent phase where they are most soluble. Choosing the correct solvent system allows for the extraction of target molecules from complex matrix components. Sometimes the process of extraction is repeated to ensure highest extraction efficiency.

The main drawbacks of LLE are related to limited solvent selectivity, which limits the use of the technique in sample preparation (especially for samples with complex nature). Limited solvent selectivity can lead to extraction of unwanted matrix interferences into the same solvent where target molecules are being extracted —e.g., lipids may be co-extracted into organic solvents with hydrophobic target analytes, or unwanted water-soluble proteins and peptides may be co-extracted into aqueous solvents together with water-soluble target analytes. Finally, difficulty in automating the process makes it more time consuming and less amenable to high-throughput applications.
Despite its drawbacks, liquid-liquid extraction has been effectively employed as a sample clean-up protocol in some tissue biomarker identification/quantification applications. Miller et al. implemented LLE as a part of clean-up protocol for extracting steroids from breast tissue homogenates, Gaikwad also employed LLE prior to UPLC-MS/MS and was able to measure over 100 different steroids in breast tissue samples, and Mousa et al. implemented an LLE procedure on a digital microfluidic chip to extract estradiol from breast tissue homogenate; the LLE led to increased estradiol signal intensity and removal of signal of interfering ions in the mass spectra of resulting tissue extractate.

1.2.2.2. Solid Phase Extraction

Solid phase extraction (SPE) is a versatile clean-up technique in which target analyte can be retained from liquid sample onto a solid sorbent according to the analyte’s and sorbent’s physical and chemical properties, with subsequent recovery of the retained analyte from the sorbent upon applying appropriate elution steps.

Depending on the nature of the analyte, different modes of SPE can be performed—for example, reversed-phase SPE employs a hydrophobic sorbent (e.g., C4, C8 or C18) and is used to retain non-polar to moderately polar analytes from a polar liquid phase, normal-phase SPE employs hydrophilic sorbents [e.g. silica (SiO\textsubscript{2}), alumina (Al\textsubscript{2}O\textsubscript{3}), magnesium silicate (MgSiO\textsubscript{3} or Florisil®), or silica modified with polar groups] and is used to retain polar analytes from a non-polar liquid phase. Ion-exchange SPE employs positively charged functional groups (e.g. quaternary ammonium-functionalized sorbents) that can retain negatively charged analytes or negatively charged sorbents (e.g. sulphonate-functionalized sorbents) that can retain positively charge analytes.

Solid phase extraction tools are commercially available in different formats suitable for small- or large- scale applications, including cartridges for preparative applications, columns for in-line SPE prior to liquid chromatography, 96-well plate formats suitable for automation, ZipTips\textsuperscript{TM} packed into pipette tips, and spin-cartridges that are suitable for microscale sample preparation.

Generally, SPE comprises four steps: (1) solid phase is pre-washed with sample matrix to condition the stationary phase (2) sample is loaded onto the solid phase by incubation, (3)
solid phase is post-washed to remove unbound undesirable sample matrix, and finally (4) sample desorption/elution in an appropriate solvent.

SPE is governed by a mechanism similar to liquid chromatographic separation mechanism, in that the equilibrium constant $K_D$ plays an important role (equation 2). In SPE, however, upon loading the sample analyte $K_D$ approaches infinity, as analyte almost completely saturates sorbent and upon analyte elution, $K_D$ approaches zero as the analyte is almost completely released into the solution phase.

$$K_D = \frac{[X]_{\text{solid phase}}}{[X]_{\text{solution}}} \quad (2)$$

where the $[X]_{\text{solid phase}}$ denotes the concentration of $X$ in the solid (or sorbent) phase, and $[X]_{\text{solution}}$ denotes the concentration of $X$ in solution.

SPE has a number of advantages over LLE including reduced extraction time, less manual intervention, ease of automation and reduced organic solvent consumption. More importantly, there is great diversity of SPE sorbents, and SPE exhibits stronger solute binding, with high $K_D$ values compared to LLE. For these reasons, SPE was used in much of the new work described in this dissertation.

1.3. Sample Detection

Textbooks can be (and are) written describing seemingly endless numbers and variations of different detection platforms that have been developed by and are used by analytical chemists. This section reviews two analytical platforms that were used for target-analyte detection in the work described in this dissertation – namely, mass spectrometry and enzyme-linked immunoassays.

1.3.1. Mass Spectrometry

Mass spectrometry (MS) is a powerful tool useful for the analysis of a wide range of analytes. MS can be employed readily for identification and quantification of ions as a function of the mass-to-charge ratio. Many interesting analytes are not ions, which has led to the development of a variety of ionization techniques. For example, MALDI and
DESI are ionization techniques that are particularly useful for imaging (see section 1.5). The ionization technique used in this dissertation is electrospay ionization (ESI), a technique that is straightforward to couple to the eluent of an HPLC separation. In ESI, sample solution is sprayed through a highly charged capillary needle (ESI capillary) at atmospheric pressure. A nebulizing gas is usually used to streamline the spraying process. Solvated positively or negatively charged droplets are produced in the ion source and a desolvation gas is applied to cause solvent evaporation of the charged droplet. Mass spectrometry offers a number of advantages when used as detection tool, including low limits of detection, a “universal” detection scheme that does not require labels or analyte modification prior to analysis, and most importantly, inherent selectivity (on the basis of mass-to-charge) is built-into the analysis. Some mass analyzers (e.g., Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR-MS) or Orbitrap) have high mass resolution, capable of distinguishing between two peaks with a small mass difference e.g. 0.001 Da. Other mass analyzers, such as the quadrupole and linear quadrupole ion trap instruments used in this dissertation have lower mass resolution (and hence reduced selectivity). But in many MS systems, a tandem (MS/MS) approach can be employed, in which an ion with particular mass-to-charge ratio is selected, which then undergoes fragmentation which can be induced by several mechanisms including but not limited to: collision induced dissociation (CID), in which ion dissociation take place through the collision of ions with neutral gas molecules, surface induced dissociation (SID), where ion dissociation occur due to collision with a solid surface and electron capture dissociation (ECD), in which multiply-charged ions capture low-energy electrons leading to charge-state reduction and subsequent fragmentation. Fragment ions then proceed to a second mass selection for evaluation; monitoring the transition between precursor ion and particular product ions can enhance confidence about a particular compound’s identification, and also improves the selectivity of the assay. For example, a particular mode of MS/MS used extensively in the work described in this dissertation is selected reaction monitoring (SRM), in which particular transitions from precursor-product ions are selectively monitored for quantification. Another advantage of the MS approach employed in my work is the ability to quantify detected compounds which have been achieved mostly using internal standards.
1.3.2. Enzyme Linked Immunoassay

The Enzyme-Linked-Immunosorbent Assay (ELISA) is antibody-antigen based test, in which the target analyte, i.e. antigen (Ag), is allowed to react to a primary antibody (Ab). Because of the high specificity of this interaction, excess/unwanted background components of the sample can be washed away after the antibody-antigen complex is formed. Enzyme-labeled antigen (for competitive ELISA) or enzyme labeled secondary antibody (for non-competitive ELISA) is then added and allowed to bind, followed by a substrate that forms an absorbant, fluorescent, or chemiluminescent product upon reaction with the enzyme. The signal intensity is directly proportional to the analyte concentration in case of non-competitive ELISAs and inversely proportional to analyte concentration for the competitive form. The non-competitive format is a powerful form of ELISA as the analyte is captured between two antibodies, which increases the selectivity of the measurement. However, if the size of the target analyte is too small, there may be no space for a binding site (epitope) of a second antibody; in this case, competitive ELISA is used. In the work described in this dissertation, I used competitive ELISAs to detect small molecules such as estradiol.

The ELISA offers a number of advantages as a detection tool including sensitivity, automation and ease of portability (less sophisticated instruments when compared to MS instruments). On the other hand "cross-reactivity" is a problem for ELISAs and immunoassays that needs to be carefully addressed through the use of proper blocking agents or by employing sample clean-up steps.

1.4. Microfluidics

Miniaturization of analytical technologies is a popular trend in life sciences, driven by the need to minimize costs and enable portability, throughput and automation. The concept of the “Micro total Analysis System or “μTAS” was introduced in 1990. Since that time, interest in this technology has grown exponentially, developing into a different basic areas of research.
Digital microfluidics (DMF) is a fluid handling technique in which droplets of samples and reagents are manipulated electrodynamically on an array of hydrophobic, insulator-coated electrodes. Two formats have been described for DMF; the closed (two plate) and open (one-plate) format. The former format allows more complex droplet handing step such as dispensing and splitting, and was used in the work described in this dissertation. Most importantly, the channel-free nature of DMF makes it particularly suitable for handling solid samples without the risk of clogging.  

In this dissertation, I present a new series of techniques relying on digital microfluidics to process milligram-size "precious" clinical tissue samples for analysis by mass spectrometry and ELISA. In developing the techniques described in Chapters 2-5, I was motivated by (a) the need for methods to handle and process small tissue samples (such samples are challenging to handle using conventional tools such as tweezers and beakers), and (b) the characteristics of DMF that are particularly useful for solid-sample processing.

When I began my doctoral work, there was only a single report of handling tissue samples on DMF.  

This study only evaluated a handful of samples, and was presented as a “proof-of-concept” analysis for identification of small molecules in droplet-scale tissue samples. In this dissertation, I describe my work improving upon the original technique, optimizing and integrating different detection modalities, as well as validating it by application to hundreds of clinical samples. I propose that this work represents a useful and important step towards the validation of DMF for analysis of small molecules from complex sample matrices, which may eventually be applied to routine biomarker testing and personalized medicine.
Chapter 2

A Microfluidic Technique for Quantification of Steroids in Core Needle Biopsies

Core needle biopsy (CNB) sampling is known to be inexpensive and minimally invasive relative to traditional tissue resectioning. But CNBs are often not used in analytical settings because of the tiny amount of sample and analyte. To address this challenge, we introduce an analytical method capable of multiplexed steroid quantification of CNB samples – those studied here ranged in weight from 2-8 mg. The new method uses digital microfluidics to extract steroids from ~mg-sized tissue samples (including a solid-phase extraction cleanup step) followed by analysis by HPLC-MS/MS. The method has limits of detection of 3.6, 1.6, 5.8 and 8.5 fmol for estradiol, androstendione, testosterone and progesterone, respectively. We propose that future generations of this method may be useful for regular quantification of steroids in core needle biopsy samples of breast tissue to inform dosage and timing of anti-hormone or hormone replacement therapies as part of a personalized medicine approach to treating a variety of hormone-sensitive disorders.

2.1. Introduction

Steroid hormones are constitutively involved in a wide range of normal physiological processes, as well as being key players in diseases like breast cancer, prostate cancer, and diabetes. In clinical laboratories, there are two principle methods used to quantify hormones: immunoassays and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Immunoassays have many advantages, including being simple, inexpensive, and sensitive; but they also suffer from disadvantages such as cross-reactivity, matrix interference, and inter-laboratory variation. The biggest drawback for immunoassays is multiplexing – a separate assay must be used for each new analyte that is measured. Methods relying on mass spectrometry, including LC-MS/MS and GC-MS/MS, also have advantages (reliability and accuracy) and disadvantages (expense, size, requirement of a trained operator), but they are well suited for multiplexing – one analysis is often sufficient to quantify many different steroid analytes.
Steroid hormones are routinely quantified in blood and serum, but there are many applications for which it is useful to evaluate hormone levels in tissue. Traditional methods for this purpose require a surgical biopsy to remove a >500 mg section of tissue, an invasive, costly process that involves a trip to the hospital, anesthesia, and risk of scarring. An attractive alternative to surgical biopsy is the core needle biopsy (CNB), in which a much smaller section (~1-10 mg) is collected in a physician’s office using biopsy needles in few minutes, without general anaesthesia and with no risk of scarring. Unfortunately, the small size of CNB samples makes them difficult to handle biochemically (or analyse). As far as we are aware, there have been no previously published reports of the quantification of steroid hormones in CNB samples.

Here we report the first method for quantifying hormones in core-needle biopsies. The method relies on digital microfluidics (DMF), a technique in which droplets of samples and reagents are manipulated electrodynamically on an array of electrodes coated with a hydrophobic insulator. The “open” format of DMF makes it particularly well suited to handling complex samples (for example, dried blood on paper) as there are no enclosed channels that might become clogged with solids. DMF was recently reported to be useful for extracting steroid hormones from tissue, blood, and serum. Here we report a significant improvement on the previous work, with innovations in sample extraction, cleanup, and derivatization, to enable the first method capable of measuring hormones in breast CNB samples. To accompany DMF sample processing, a custom LC-MS/MS method was developed to allow for the simultaneous quantification of estradiol (E2), androstenedione (AD), testosterone (TS), and progesterone (PG). We propose that this technique and variations thereof may eventually be useful for personalized medicine strategies in which hormone levels are regularly measured to inform dosage and timing of anti-hormone or hormone replacement therapies. More generally, we propose that these techniques may be useful for a wide range of applications that would benefit from quantification of steroid hormones and other small molecules in CNB samples.
2.2. Experimental

2.2.1. Reagents and Materials

Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON), including 1,3,5-Estratriene-3,17β-diol (estradiol, E2) with purity of 98% and 1,3,5-Estratriene-3,17β-diol-16,16,17-d3 (estradiol-d3, E2d3) with an isotopic purity of 98%. 4-androsten-3,17-dione (androstenedione, AD) with purity of >98%, 4-androsten-3,17-dione-2,2,4,6,6,16,16-d7 (deuteroandrostenedione-d7, ADd7) with an isotopic purity of >98%, 4-androsten-3-17β-ol-3-one (testosterone, TS) with purity of >98%, 4-androsten-3-17β-ol-3-one-16,16,17-d3 (deuterotestosterone-d3, TSd3) with an isotopic purity of >98%, 4-pregnen-3,20-dione, (progesterone, PG) with purity of >98% and 4-pregnen-3,20-dione-2,2,4,6,6,17α,21,21,21-d9 (deuteroprogesterone-d9, PGd9) with an isotopic purity of >98% were purchased from Steraloids (Newport, RI). Parylene-C dimer was from Specialty Systems (Indianapolis, IN), and Teflon-AF was obtained from DuPont (Wilmington, DE). All solvents were HPLC grade (>99.9% purity), and all other chemicals used were of the highest grade available (>98% purity).

For each hormone, a stock solution (1 mg/mL) was prepared in methanol. Working solutions of each standard (10 µg/mL) were formed in methanol by serial dilution. Two stock mixtures were prepared from the working solutions in methanol: Mixture A contained the four standard hormones (E2, AD, TS, and PG, 200 ng/mL ea.), and Mixture B contained the four deuterated hormones (E2d3, ADd7, TSd3, PG and PGd9, 200 ng/mL ea.). All stock and working solutions were stored at −20°C.

A C12 casting solution was prepared by mixing 279 µL of butyl acrylate (99%), 150 µL of 1,3-butanediol diacrylate (98%), 69 µL of lauryl acrylate (90%), 2.5 mg of 2,2-dimethoxy-2-phenylacetophenone (99%), and 1 mL of porogen comprising a 4:1:1 ratio of acetonitrile, 95% ethanol, and 5 mM phosphate buffer at pH 6.8.
2.2.2. DMF Device Fabrication, Assembly, and Operation

Two-plate digital microfluidic devices were fabricated in the University of Toronto Nanofabrication Centre (TNFC), using microfabrication techniques described previously.\textsuperscript{126} Briefly, top plates were formed from indium tin oxide (ITO)-coated glass substrates (Delta Technologies Ltd., Stillwater, MN) coated with 50 nm Teflon-AF, and bottom plates were formed from chromium-coated glass substrates (Telic, Valencia, CA) coated with 7 μm Parylene-C and 50 nm Teflon-AF. Bottom plates featured an array of 80 actuation electrodes (2.2 × 2.2 mm each) connected to 8 reservoir electrodes (16.4 × 6.7 mm each). The actuation electrodes were roughly square with interdigitated borders (140 μm peak-to-peak sinusoids), with inter-electrode gaps of 30–80 μm. Devices were assembled by joining a top and bottom plate with a spacer formed from five pieces of 3M Scotch double-sided tape (St. Paul, MN) with a total spacer thickness of 450 μm.

The automated open-source DropBot DMF control system (described in detail elsewhere\textsuperscript{127}) was used to control droplet movement on chip. Aliquots of reagents were loaded onto a DMF device by pipetting a droplet onto the bottom plate at the edge of the top plate, and simultaneously applying sine wave voltages (\(\sim 100\) Vrms, 10 kHz) between the top plate electrode and successive electrodes on the bottom plate via a custom pogo-pin connector (comprising an array of 120 pogo pins on a printed circuit board, each pin wired to solid-state switches in the DropBot system\textsuperscript{127}), to draw the fluid into the reservoir and further manipulate droplet movement. Droplet operation was monitored and recorded with a webcam (LifeCam Studio with frame rate of 30 fps, Microsoft Mississauga, ON).

2.2.3. Porous Polymer Monolith Formation

Porous polymer monoliths (PPMs) were prepared by photopolymerization of a C12 casting solution. 100 μL of the casting solution was sandwiched between two pieces of unpatterned glass slides coated with 50 nm Teflon-AF separated by 450 μm spacers. Polymerization was initiated by exposure to UV radiation (100 W, 365 nm, 5 min).\textsuperscript{128} The resulting bulk PPM substrates were \(\sim 50\) mm diameter, 450 μm high, and had masses
of ~ 30 mg. 2.5 mm diameter PPM discs were separated from the larger substrates using a manual biopsy punch (Miltex, York, USA) and then manually positioned onto DMF devices as described previously.129

2.2.4. Rat samples

Rat fat samples were obtained from 12-month old rats at the animal facility in the Samuel Lunenfeld Research Institute at Mount Sinai Hospital. Rats were euthanized without anesthesia using CO₂ for ~4 min; tissue specimens were collected from abdominal region with scissors and were stored at -80°C until use. Prior to analysis tissue specimens were thawed, sectioned, and weighed (samples ranged from 3.6-6.3mg for DMF-scale and 96-113 mg for macro-scale sample processing experiments, respectively), prior to placing them in microcentrifuge tubes. DMF-scale specimens were homogenized by manual grinding with a disposable polypropylene pestle (VWR, ON, Canada), while on dry ice, samples were then kept on dry ice until analysis.

Each sample was then spiked with exogenous hormones by pipetting aliquots of dilutions of mixture A in methanol onto the tissue and allowing it to dry at room temperature (~30 min). For experiments evaluating long-term signal stability and extraction time, the spiking solution was a 25 µL aliquot of a dilution of mixture A (5 ng/mL of each analyte). For experiments evaluating extraction efficiency, the spiking solution was a 2.5 µL aliquot (for DMF experiments) or a 20 µL aliquot (for macro-scale experiments) of a dilution of mixture A (100 ng/mL of each analyte). As a control, some samples were spiked with identical volumes of neat methanol containing no analytes.

2.2.5. Human Samples

Ethical approval for the study was granted by the Research Ethics Board of Mount Sinai Hospital (Reference number 07-0015A). Written informed consent was obtained from each participant before enrollment. Breast tissue samples were collected from four different groups of patients: (1) premenopausal women, (2) postmenopausal women [at least 12 months since the last menstrual period that have never taken menopausal hormone therapy (HT)], (3) postmenopausal women on HT (1.0 mg/day 17ß-estradiol,
Shire Canada Inc, Saint-Laurent, QC), and (4) postmenopausal women on HT + aromatase inhibitor (AI) therapy (2.5 mg/day letrozole, Novartis Canada, Dorval, Quebec). The samples were collected using Quick-Core Biopsy Needles (QC-16-6.0-20T, Cook Medical, USA) after local intradermal injection of 0.5 mL of 2% Xylocaine (AstraZeneca, Wilmington, DE). All samples were taken from the right breast at the 10 o'clock position, 5 cm from the border of the areola. The samples were stored in centrifuge tubes at -80°C until use. Prior to analysis, specimens were thawed, transferred to microcentrifuge tubes, and weighed, followed by homogenization by manual grinding with a disposable polypropylene pestle (VWR, ON, Canada), while on dry ice. Samples were then kept on dry ice until analysis.

2.2.6. DMF Sample Processing

For each experiment, a tissue homogenate and a PPM disc were positioned on a DMF bottom plate. 25 µL of lysing solvent (dichloromethane:acetone 80:20) was pipetted onto the sample and allowed to evaporate (~30 s), and then the device was assembled with a top plate. 25 µL of a dilution of mixture B (E2d3, ADd7, TSd3, PG and PGd9, 2 ng/mL ea.) was loaded into a reservoir and driven onto the tissue sample. The droplet was moved across the tissue sample in a clock-wise circular motion for 5 min, incubated statically for 10 min, and the sample-extract droplet was driven away from the tissue sample and delivered to the PPM disc for clean-up. Prior to this step, the PPM disc was activated by dispensing a droplet of methanol, delivering it to the PPM disc, incubating for 5 min, and driving the remaining methanol to waste. The sample-extract droplet was actuated back and forth across the PPM disc for 2 min before it was driven away to a collection reservoir.

The process described above was repeated for four times, generating four separate extract-droplets, which were typically pooled and allowed to dry at room temperature for ~30 min. In some experiments, each extract droplet was collected and analyzed individually. The top plate was removed, and the dried samples were reconstituted in a 25 µL aliquot of a 1:1 mixture of 1 mg/mL dansyl chloride in acetone and 100 mM sodium bicarbonate (pH 10.7). This solution was transferred to a capped vial and incubated in a
60°C water bath for 5 min, then mixed with 25 µL of 50:50 methanol:DI water and transferred into a 96-well plate for analysis.

2.2.7. Macro-Scale Sample Processing

Macro-scale tissue samples (spiked and unspiked with exogeneous analytes, as described above) were processed according to an adaptation of a previously reported method. Briefly, each sample was ground and suspended in a 20 µL aliquot of a dilution of mixture B (20 ng/mL of each deuterated hormone). A 4 mL aliquot of methanol was added, and the resulting suspension was mechanically homogenized and centrifuged (1,200 × g) for 5 min. The supernatant was transferred to a new tube and evaporated to dryness under a stream of nitrogen at 40°C. Each dried sample was reconstituted in a 200 µL aliquot of a 1:1 mixture of 1 mg/mL dansyl chloride in acetone and 100 mM sodium bicarbonate (pH 10.7). This solution was incubated in a 60°C water bath for 5 min in a capped vial, then mixed with 200 µL of 50:50 methanol:DI water and finally transferred into a HPLC vial for analysis.

2.2.8. HPLC-MS/MS

A QuattroMicro triple quadrupole mass spectrometer (Waters, Milford, MA) was operated in multiple reaction monitoring (MRM) mode using MassLynxv4.0 (Waters). Electrospray ionization (ESI) was used in positive ion mode. The capillary voltage was 3.5 kV, the extractor cone voltage was 3 V, and the detector voltage was 650 V. The cone voltage ranged from 30-50 V (Table 2.1). Cone gas flow was 30 L/h, and desolvation gas flow 600 L/h, and the source temperature and desolvation temperatures were 100 and 400°C, respectively.

Chromatographic separations were performed using an Agilent Technologies 1200 series HPLC system (Santa Clara, California). The column was an Agilent Zorbax Eclipse Plus C18 column (2.1 mm i.d. x 100 mm long, 1.8 µm particle dia.) protected by a C18 Zorbax guard column (2.1 mm i.d. x 12.5 mm long, 5 µm particle dia., Agilent) and an in-line filter (2.1 mm dia., 0.2 µm pore dia., Agilent). The HPLC was operated at ambient temperature in gradient elution mode at a flow rate of 0.1 mL/min. The autosampler
injection volume was 20 μL. The gradient started with 50% mobile phase A (0.1% formic acid in DI water) and 50% mobile phase B (0.1% formic acid in methanol), changed linearly to 80% mobile phase B over 2 min, and then changed linearly again to 100% mobile phase B over the next 2 min. Mobile phase B was then held at 100% for 5 min before linearly decreasing back to 50% over 2 min and holding at 50% for 11 min for a total runtime of 22 min.

2.2.9. Calibration Curves and Quantitative Analysis

HPLC-MS/MS conditions were optimized using dilutions of hormone mixtures A and B in 50:50 methanol:DI water. Briefly, dilutions of non-deuterated standards at 20, 50, 100, 500, 2000 and 5000 pg/mL were formed from mixture A containing 1000 pg/mL of deuterated standards (from mixture B). MRM transitions of each analyte and labeled internal standard, as well as ionization conditions, collision energies and retention time windows are listed in Table 2.1.

Table 2.1. HPLC-MS/MS with multiple reaction ion monitoring (MRM) conditions for eight deuterated and non-deuterated hormones.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time window (min)</th>
<th>MRM transition</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>8.5-10.2</td>
<td>287/97</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>287/107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADd7</td>
<td>8.5-10.2</td>
<td>294/100</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>294/113</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS</td>
<td>8.9-10.3</td>
<td>289/97</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>289/109</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSd3</td>
<td>8.9-10.3</td>
<td>292/97</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>292/109</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>9.75-11.25</td>
<td>315/97</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>315/109</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGd9</td>
<td>9.75-11.25</td>
<td>324/100</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>324/113</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dansylated E2</td>
<td>12.2-13.6</td>
<td>506/171</td>
<td>50</td>
<td>35</td>
</tr>
<tr>
<td>Dansylated E2d3</td>
<td>12.2-13.6</td>
<td>509/171</td>
<td>50</td>
<td>35</td>
</tr>
</tbody>
</table>
Calibration curves were constructed by plotting the ratio of the area under the curve (AUC) for analyte product ions relative to those of their internal standards as a function of concentration. Regression lines were fit to the data, and limits of detection (LODs) were determined as the analyte concentrations corresponding to the signal of the sample blank plus three standard deviation of the blank value. A new calibration curve was generated before and after each tissue analysis (rat or human) for quality control (QC) to evaluate instrument performance and to assess errors related to analytical variation. The average calibration data from the two QC plots (collected before and after the sample) were used to quantify the extracted hormones in each tissue sample.

2.3. Results And Discussion

2.3.1. HPLC-MS/MS Analysis of Steroid Hormones

The primary goal of this work was to develop means to quantify steroid hormones in core needle biopsy (CNB) tissue samples. Four hormones, estradiol (E2), androstenedione (AD), testosterone (TS), and progesterone (PG), were chosen as test candidates, given the importance of these analytes in breast cancer. AD, TS, and PG are readily ionisable by ESI and thus are compatible with HPLC-MS/MS analysis of trace amounts of analyte. In contrast, the ionization efficiency of E2 (even in negative ESI mode) is relatively low because of its phenolic functional group. To improve ionization efficiency of E2, we developed a method relying on derivatization with dansyl chloride following sample extraction, where the phenolic group of E2 is modified with a dansyl moiety bearing a readily ionizable secondary amine. Figure 2.1 demonstrates the difference in signal intensity observed for (1) dansylated E2 in positive mode and (2) underivatized E2 in negative mode. As shown, the signal intensity of dansylated E2 is ~30 times higher than that for underivatized E2. The strong signal of dansylated E2 allowed for the development of a sensitive method with low limit of detection (described below).

While dansylation of estradiol has been reported previously, it has only been used in applications in which the analytes to be evaluated were all estrogens (i.e., E2, estrone,
and estriol), not a mixture of estrogens, progesterone, and androgens (as desired for this work). Importantly, the dansylation derivatization procedure was found to not affect the signals for AD, TS, and PG. In the work reported here, the E2 derivatization was implemented off-chip (to avoid problems associated with evaporation of acetone at elevated temperature), but in the future, we propose to implement this step on-chip in a miniature sealed chamber (as described previously\textsuperscript{139}) for a completely automated method.

**Figure 2.1.** Sensitivity improvement for measurement of estradiol by derivatization with dansyl chloride. (The LC-MS/MS conditions were optimized for the detection of estradiol with or without derivatization, respectively.) (A) Multiple reaction monitoring (MRM) chromatogram of the 271/145 MRM transition for estradiol (20 pg/mL) in negative ion mode (the inset is a magnified reproduction of the peak). (B) MRM chromatogram of the 506/171 MRM transition for dansylated estradiol (20 pg/mL) in positive ion mode. *Total ion chromatogram intensity for (A) has been scaled to that of (B) for better data visualisation.
Armed with a method for selective derivatization of E2, an HPLC-MS/MS method was developed for simultaneous quantification of the four analytes. The method was validated by running a series of dilutions of a mixture of native hormones (mixture A) containing deuterated internal standards at 1000 pg/ml (mixture B) as described in the methods section. The ratio of area under curve (AUC) for each standard relative to its deuterated IS was plotted as a function of standard concentration, and the results are described in Table 2.2. Good linearity was observed for all four analytes; linear regression equations were generated with $R^2$ values ranging from 0.9967 to 0.9997 for all of the hormones. The limits of detection (LODs) were 3.6, 1.6, 5.8, and 8.5 fmol for E2, AD, TS and PG, respectively.

**Table 2.2.** Calibration data for four steroid hormones.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Slope</th>
<th>Intercept</th>
<th>$R^2$</th>
<th>Intraday CV (%)</th>
<th>Interday CV (%)</th>
<th>LOD (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dansylated E2</td>
<td>0.0153</td>
<td>0.113</td>
<td>0.9997</td>
<td>1.3</td>
<td>1.6</td>
<td>3.6</td>
</tr>
<tr>
<td>AD</td>
<td>0.0156</td>
<td>0.086</td>
<td>0.9967</td>
<td>7.5</td>
<td>5.0</td>
<td>1.6</td>
</tr>
<tr>
<td>TS</td>
<td>0.0137</td>
<td>0.018</td>
<td>0.9996</td>
<td>4.2</td>
<td>7.5</td>
<td>5.8</td>
</tr>
<tr>
<td>PG</td>
<td>0.0305</td>
<td>0.116</td>
<td>0.9988</td>
<td>5.3</td>
<td>6.0</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Calibration plots were repeatedly generated on multiple days (and with multiple operators), and the mean interday and intraday precision was 4.6 and 5.0% RSD, with a limit of quantification (LOQ) of 13.2, 5.4, 20.2 and 27.0 fmol for E2, AD, TS and PG, respectively. Five such plots collected over the span of five weeks (during which tissue samples were evaluated, as described below) are shown in Figure 2.2. As shown, the bias in slope was less than 10% for all analyses, with the highest agreement for the (most relevant) lower concentration range (as is often the case in such analyses\textsuperscript{140}). In practice,
for each tissue sample analyzed, a separate calibration plot was collected before and after analysis as a measure of quality control.

**Figure 2.2.** HPLC-MS/MS calibration plots for derivatized estradiol, androstenedione, testosterone, and progesterone obtained in week 1 (blue diamonds), 2 (red squares), 3 (purple Xs), 4 (blue asterisks), and 5 (orange circles).

There have been several methods reported previously\(^{100,141-151}\) for the analysis of steroid hormones by HPLC-MS. There are only four previous reports (those of Guo et al.,\(^ {141}\) Koal et al.,\(^ {142}\) Kinoshita et al.,\(^ {151}\) and Gaikwad\(^ {100}\)) of methods capable of quantifying the four analytes described here (E2, AD, TS, and PG) simultaneously. These techniques are useful, but they require large sample volumes (200 µL,\(^ {141}\) 500 µL,\(^ {142}\) 1 g,\(^ {151}\) and 200 mg\(^ {100}\)), making them a poor match for analyzing very small samples. The new method reported here was specifically designed to be compatible with microfluidic-processed ~5 mg core needle biopsy tissue samples, as described below.
2.3.2. On-Chip Extraction and Clean-up

A digital microfluidic device (Fig. 2.3A) was designed for extraction of hormones from ~mg-size tissue samples and in-line sample cleanup. In a typical experiment, a tissue sample is placed onto a device, a droplet of extraction solvent (neat methanol containing internal standards) is driven onto the tissue sample, and then incubated to extract the exogenous analytes (Fig. 2.3B-C). After extraction, the droplet is driven to a porous polymer monolith (PPM) disc to remove unwanted non-polar constituents by solid phase extraction (SPE) (Fig. 2.3D-E), and then the purified sample is driven to a collection reservoir. This extraction and cleanup process is repeated multiple times, and the pooled, processed sample is collected for off-line dansylation and analysis by HPLC-MS/MS. In practice, the process requires < 2 hr from sample to analysis; in the future, the extraction and purification might be implemented in parallel (as has been reported for DMF dried blood spot analysis\textsuperscript{125}) to improve throughput.

A series of ~ 5 mg samples of abdominal rat fat with spiked with exogenous E2, AD, TS, and PG (designed to mimic CNB samples from human breast tissue) were used to optimize the method shown in Figure 2.3. Three key parameters were evaluated: long-term signal stability, extraction time, and extraction efficiency. For the first parameter (long-term signal stability), in initial tests with rat fat samples, it was observed that analyte peak intensities decreased steadily over time (on the scale of days-weeks) if samples were evaluated directly after extraction (with no SPE purification step). We hypothesized that this was caused by ion suppression and instrument fouling from tissue constituents other than the analytes. To circumvent this problem, a SPE process was developed (Fig. 2.3D-E), building on recent reports of the use of porous polymer (PPM) discs\textsuperscript{128,129} for DMF-SPE. Note that the method used here is different from “standard" SPE techniques (and those reported previously\textsuperscript{24,25}) in that the analytes remain in solution, and the putative interferants (e.g., endogenous amines, carbohydrates, or lipids) are retained by the solid matrix, which bears C12 moieties.
Figure 2.3. Digital microfluidic extraction/cleanup from tissue samples. Schematic of the device (A) and frames from a movie depicting extraction from tissue sample (B-C) and cleanup on a porous polymer (PPM) disc for solid phase extraction (SPE) (D-E).

Mass spectra demonstrating the effect of the cleanup procedure are shown in Figure 2.4. The addition of the SPE step has modest effect on analyte peak heights (labeled 1, 2, and 3), but significantly reduces the peak heights of a series of unidentified interferants (m/z 400-700). Most importantly, upon inclusion of the SPE step into the standard analysis method, mass spectral signal intensities were observed to be stable for several weeks of replicate analyses.
**Figure 2.4.** Sample cleanup. Rat tissue samples were spiked with 1 µg/mL AD, TS and PG. Representative direct infusion MS spectra of rat tissue samples extracted by DMF after (above, red) or before (below, green) an additional on-chip cleanup by solid phase extraction (SPE). Analyte peaks are labeled as 1 (AD), 2 (TS), and 3 (PG), and their peak heights changed only modestly (i.e. within 10%) after SPE cleanup. In contrast, the peak intensities of a series of unidentified interferants (dotted blue box, m/z 400-700) are significantly reduced after SPE cleanup.

For the second parameter (extraction time), samples spiked with exogenous analytes were sequentially extracted in one, two, three, and four droplets of extraction solvent (each with 15 min incubation, as described in the methods section). As shown in Figure 2.5, for each of the analytes, the amount of signal (measured as the ratio of AUC for standard relative to deuterated I.S.) decreased for each successive droplet, approaching the level of the blank in the fourth extraction. Thus, in all subsequent experiments, four successive extractions (representing a total of 1 hour of incubation) were performed and pooled prior to analysis.
Figure 2.5. Extraction time. HPLC-MS/MS MRM chromatogram area under curve (AUC) ratios of standard (spiked in ~5 mg rat tissue samples spiked with 25 µL of 5 ng/mL of each hormone) to deuterated IS (dissolved in extraction solvent) for (A) dansylated E2 (blue diamonds), (B) AD (red triangles), (C) TS (orange circles), and (D) PG (black crosses) after extraction from rat tissue samples by DMF. Data are plotted as a function of extraction number, where each number represents one of four sequential droplets (i.e., droplets 1, 2, 3, and 4) used to extract analytes from a given sample in succession. Each condition was repeated 3 times, and error bars represent ±1 S.D. The black dotted lines represent the AUC ratios of blanks.
For the third parameter (extraction efficiency), in an initial experiment, extracts of nonspiked rat fat samples were generated on chip (as above) and were evaluated using the calibration data (Table 2.2). The extracts were found to contain undetectable endogenous levels of E2, AD, and TS, but high concentrations (above the calibration range) of endogenous PG. Thus, the samples were deemed to be a good model for evaluating extraction efficiency of E2, AD, and TS, but not PG (in the future, a different type of tissue should be used to extract and quantify PG). A series of tissue samples was then spiked with exogenous E2, AD, and TS, and the recovered concentrations were determined using the calibration data (Table 2.2). The ratios of recovered/extracted amounts to spiked amounts (i.e., extraction efficiency) were determined after subtracting background levels. For comparison, large sections (~100 mg ea.) of identical tissue samples were processed off-chip using conventional techniques. The extraction efficiencies of the DMF method were 65.3%, 80.3% and 89.1% (with CVs of 22.5, 21.4 and 11.2) for E2, AD and TS, respectively. These values were comparable to those of the macro-scale technique which had recoveries of 101.7, 79.8, and 102.9 and CVs of 3.7, 27.6 and 18.9 for E2, AD, and TS, respectively. The similarities are remarkable given the differences in size and technique (e.g., micro-scale samples were not homogenized or centrifuged while macro-scale samplers were extensively homogenized and then centrifuged) for the two methods.

The extraction efficiencies described above for the DMF method were deemed acceptable for E2, AD, and TS, and were used to correct values obtained for human CNB samples (below). Remaining (unextracted) analytes are likely retained in adipose cells and tissue debris. In the future (if necessary), more aggressive cell lysis techniques might be adopted in the DMF workflow, perhaps employing chemical digestion.

2.3.3. Analysis of Human CNB Samples

The DMF-HPLC-MS/MS method described above was applied to thirty-two CNB breast tissue samples. These samples were the first evaluated from a large (and on-going) study designed to evaluate the effects of aromatase inhibitor (AI) therapy and menopause hormone therapy (HT) on local and systemic hormone concentration in pre- and post-
menopausal women. The samples tested here were obtained from four different groups of subjects, including (1) four samples from premenopausal subjects not undergoing therapy, (2) twelve samples from postmenopausal subjects not undergoing therapy, (3) seven samples from postmenopausal subjects undergoing a combination of AI/HT, and (4) nine samples from postmenopausal subjects undergoing HT. The results were normalized to the mass of the samples and are listed in Table 2.3.

As shown in Table 2.3, a “raw” result (i.e., the amount measured) for each analyte is listed for each sample. In an ideal case, standard addition analysis (comprising splitting each sample and spiking different amounts of analyte) would be used to convert the raw value to an actual concentration in the sample; unfortunately, CNB samples are too small to split and analyzed by standard addition. Thus, in the work described here, “corrected” values for E2, AD, and TS were extrapolated using the extraction efficiencies determined from rat tissue samples as a proxy (Fig. 2.6). For PG (the only analyte for which an extraction efficiency could not be determined in rat tissue samples), only the raw result is listed. In future work, human breast tissue samples will be obtained to measure extraction efficiencies in samples that more closely match the matrix of CNB samples. But we propose that the technique used here (with correction from rat tissue measurements) is sufficient to confirm that this type of analysis is possible.
Table 2.3. Measured hormone amounts in core needle biopsy (CNB) breast tissue samples from human subjects normalized by sample mass. Raw values are the measured amounts; corrected values are extrapolated from the extraction efficiencies (Fig. 2.6)* for E2, AD, and TS, respectively. The subjects were from four groups: premenopausal with no treatment (group 1: 1-4), postmenopausal with no treatment (group 2: 5-16), postmenopausal with HT/AI (group 3: 17-23), and postmenopausal with HT only (group 4: 24-32).

<table>
<thead>
<tr>
<th>CNB sample #</th>
<th>E2 (fmol/mg)</th>
<th>AD (fmol/mg)</th>
<th>TS (fmol/mg)</th>
<th>PG (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw</td>
<td>Corrected</td>
<td>Raw</td>
<td>Corrected</td>
</tr>
<tr>
<td>1</td>
<td>10.0</td>
<td>15.35</td>
<td>12.5</td>
<td>15.51</td>
</tr>
<tr>
<td>2</td>
<td>4.8</td>
<td>7.36</td>
<td>17.1</td>
<td>21.30</td>
</tr>
<tr>
<td>3</td>
<td>4.6</td>
<td>7.01</td>
<td>5.0</td>
<td>6.20</td>
</tr>
<tr>
<td>4</td>
<td>5.4</td>
<td>8.32</td>
<td>8.0</td>
<td>10.01</td>
</tr>
<tr>
<td>5</td>
<td>6.4</td>
<td>9.87</td>
<td>11.3</td>
<td>14.06</td>
</tr>
<tr>
<td>6</td>
<td>21.5</td>
<td>33.02</td>
<td>5.9</td>
<td>7.35</td>
</tr>
<tr>
<td>7</td>
<td>22.9</td>
<td>35.06</td>
<td>8.5</td>
<td>10.56</td>
</tr>
<tr>
<td>8</td>
<td>11.6</td>
<td>17.75</td>
<td>5.6</td>
<td>6.94</td>
</tr>
<tr>
<td>9</td>
<td>7.8</td>
<td>11.99</td>
<td>13.4</td>
<td>16.69</td>
</tr>
<tr>
<td>10</td>
<td>6.4</td>
<td>9.87</td>
<td>2.5</td>
<td>3.09</td>
</tr>
<tr>
<td>11</td>
<td>3.9</td>
<td>5.95</td>
<td>4.5</td>
<td>5.54</td>
</tr>
<tr>
<td>12</td>
<td>25.5</td>
<td>39.02</td>
<td>4.0</td>
<td>4.99</td>
</tr>
<tr>
<td>13</td>
<td>7.0</td>
<td>10.75</td>
<td>1.6</td>
<td>2.03</td>
</tr>
<tr>
<td>14</td>
<td>26.7</td>
<td>40.90</td>
<td>8.2</td>
<td>10.16</td>
</tr>
<tr>
<td>15</td>
<td>4.6</td>
<td>7.07</td>
<td>2.8</td>
<td>3.45</td>
</tr>
<tr>
<td>16</td>
<td>19.4</td>
<td>29.73</td>
<td>4.5</td>
<td>5.59</td>
</tr>
<tr>
<td>17</td>
<td>56.6</td>
<td>86.82</td>
<td>1.8</td>
<td>2.28</td>
</tr>
<tr>
<td>18</td>
<td>47.0</td>
<td>72.07</td>
<td>4.2</td>
<td>5.24</td>
</tr>
<tr>
<td>19</td>
<td>15.9</td>
<td>24.30</td>
<td>1.0</td>
<td>1.28</td>
</tr>
<tr>
<td>20</td>
<td>12.0</td>
<td>18.38 &lt; LOD</td>
<td>&lt; LOD &lt; LOD</td>
<td>2.3</td>
</tr>
<tr>
<td>21</td>
<td>1.1</td>
<td>1.70</td>
<td>0.9</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>22</td>
<td>11.0</td>
<td>16.89</td>
<td>2.3</td>
<td>2.88</td>
</tr>
<tr>
<td>23</td>
<td>18.1</td>
<td>27.75</td>
<td>9.6</td>
<td>11.97</td>
</tr>
<tr>
<td>24</td>
<td>4.8</td>
<td>7.40</td>
<td>2.9</td>
<td>3.59</td>
</tr>
<tr>
<td>25</td>
<td>10.5</td>
<td>16.13</td>
<td>7.3</td>
<td>9.08</td>
</tr>
<tr>
<td>26</td>
<td>6.0</td>
<td>9.21</td>
<td>9.2</td>
<td>11.45</td>
</tr>
<tr>
<td>27</td>
<td>5.3</td>
<td>8.16</td>
<td>4.7</td>
<td>5.80</td>
</tr>
<tr>
<td>28</td>
<td>9.5</td>
<td>14.49</td>
<td>6.4</td>
<td>7.93</td>
</tr>
<tr>
<td>29</td>
<td>7.4</td>
<td>11.38</td>
<td>5.1</td>
<td>6.29</td>
</tr>
<tr>
<td>30</td>
<td>10.7</td>
<td>16.45</td>
<td>2.4</td>
<td>3.00</td>
</tr>
<tr>
<td>31</td>
<td>13.1</td>
<td>20.11</td>
<td>2.2</td>
<td>2.73</td>
</tr>
<tr>
<td>32</td>
<td>24.9</td>
<td>38.20</td>
<td>2.4</td>
<td>2.95</td>
</tr>
</tbody>
</table>

*DMF method precision (based on data obtained from processing rat fat tissue samples) was 22.5, 21.4 and 11.2% RSD for E2, AD and TS respectively.
Figure 2.6. Extraction efficiency. The analyte recoveries for E2, AD, and TS in spiked rat tissue were determined using the DMF technique (red) and a conventional macro-scale method (blue). Each sample (~100 mg for macroscale and ~5 mg for DMF) was spiked with a mixture of exogenous E2, AD, and TS prior to extraction. The volume and concentration of the spiking solutions (2.5 µL/100 ng/mL standards for DMF and 20 µL/100 ng/mL standards for macro-scale) were selected such that perfect extraction would yield a 5 ng/mL concentration of each analyte in the final (extracted, reconstituted) solutions that were analyzed. Analyte concentrations in non-spiked tissue samples were measured and the average values were subtracted from those determined from spiked samples. These values were expressed as percent recoveries relative to spiked amounts. Error bars represent ± 1 S.D. for averages obtained from three independent tissue samples.
A one-way ANOVA was conducted to compare the corrected values of E2, AD and TS (and the raw values of PG) for the four experimental groups. For AD and TS, the ANOVA differences were significant, and individual t-tests were performed to identify the affected groups. For AD, the average value for group (1) 13.3 ± 6.58, was significantly greater than the average value for group (2) 7.54± 4.51 (p<0.05). For TS, the average value for group (2) 19.4± 12.4, was significantly greater than the average value for group (3) 4.47 ± 4.04 (p<0.01), and group (4) 4.55±3.05 (p<0.01). No statistical differences were observed for E2 or PG. In general, the sample size numbers for each of the groups described here is small, so any potential conclusions that might be drawn are tenuous. Regardless, these data prove the principle that measuring hormone levels in CNB samples is possible, and opens the door for future studies with larger sample sets.

When evaluating the entire group of samples tested here (n = 32), the ratios between the average raw measured levels of hormones, roughly 2:3:4:5 for AD:TS:PG:E2 (all in the same order of magnitude), are quite different than the relative levels reported\textsuperscript{152,153} for the same hormones in circulation (serum) in similar patient groups. This suggests that the assumption that circulating hormone levels (which are routinely measured) are replicated in tissue (which is not routinely measured) is a poor one. The hormone levels in breast tissue are particularly important for patients undergoing AI therapy and/or HT; thus, we propose that in the future, variations on the methods described here may be useful for a personalized medicine approach to dosing and timing of these therapies.

2.4 Conclusion

We report the first technique capable of quantification of steroid hormones in core needle biopsy samples, relying on digital microfluidics, solid phase extraction, analyte derivatization, and HPLC-MS/MS. We propose that this type of method will be useful in the future for a wide range of applications, including personalized approaches to diagnosing and treating hormone-sensitive cancers.
Chapter 3

The Effect Of Bilateral Salpingo-Oophorectomy On Circulating And Breast Tissue Hormone Levels, A Digital Microfluidics Approach

Prophylactic bilateral oophorectomy (surgical removal of the ovaries) among women with a BRCA1 or BRCA2 mutation has been shown to reduce the risk of breast cancer. It is not clear whether this risk reduction is directly associated with reduction in levels of circulating steroids or indirectly through lowering concentration of circulating androgens which may serve as precursors for local synthesis of tissue estrogens. Clarification of the mechanism would help to develop recommendations regarding chemoprevention, and to establish an endocrine basis for this observation. In Chapter 2, we have introduced a steroid quantification platform based on digital microfluidics- liquid chromatography-tandem mass spectrometry (DMF-LC-MS/MS), that allows for simultaneous quantification of steroid hormones in core-needle-biopsies (CNB). The technique can handle CNBs (milligram-size tissue samples) and effectively extract and quantify steroids within the droplet-scale tissue samples with high precision and good sensitivity comparable to macroscale tissue processing techniques. In this work, we use this technique to quantify four steroids, E2, AD, TS and PG, from paired patient samples before and after oophorectomy in an attempt to understand the role of the surgery in endogenous steroid hormone levels.

3.1. Introduction

Breast cancer remains the most common malignancy diagnosed among women in Canada and the United States and the second leading cause of cancer death in women. Experimental and observational studies have consistently demonstrated that sex hormones play an important role in breast carcinogenesis. Estrogens bind and activate estrogen receptors in breast tissue cells, initiating a cascade of events that lead to cell proliferation and contribute to tumor growth which may also lead to accumulation of genetic mutations. Androgens have also been hypothesized to increase breast cancer risk either directly by increasing cellular growth and proliferation, or indirectly by their
conversion to estrogen by the action of aromatase enzyme.\textsuperscript{156} Emerging evidence also support a role of progesterone (PG)-signaling on the pathogenesis of breast cancer, combined hormone replacement therapy (i.e., estrogen plus progesterone) has been associated with an increased risk of developing breast cancer\textsuperscript{157} and \textit{in vivo} studies have provided mechanistic evidence to explain these observations and strongly implicating the receptor activator of nuclear factor κB (RANK)/RANK ligand (RANKL) signalling pathway in breast cancer development.\textsuperscript{158-162}

Epidemiologic risk factors include reproductive and hormonal factors that increase exposure of the breast to the mutagenic and mitogenic effects of hormones; while protective factors include those that limit lifetime exposures.\textsuperscript{163,164}

There is substantial evidence strongly supporting an increased risk of breast cancer with elevated circulating estrogen (e.g., estradiol [E2], estrone) and androgen (e.g., testosterone [TS], dehydroepiandrosterone sulfate [DHEAS]) levels in the general population.\textsuperscript{165} Among postmenopausal women, there is also a significant positive relationship between cancer risk with prolactin and progesterone (PG) circulating levels, but an inverse relationship between cancer risk and sex hormone binding globulin (SHBG); these hormone markers are generally not associated with risk in premenopausal women.\textsuperscript{166-171} Interestingly, studies of circulating progesterone [PG] have generally reported no association with premenopausal breast cancer (levels are too low in postmenopausal women\textsuperscript{170}) while randomized placebo-controlled trials of hormone replacement therapy have clearly demonstrated an increased risk with combined estrogen and progesterone (E2+PG) and no effect with estrogen alone formulations.\textsuperscript{172,173}

Much of the evidence for hormonal breast carcinogenesis comes from clinical and epidemiologic studies of circulating or urinary estrogen and other hormones,\textsuperscript{165} rather than from measurements of hormone level in tissue of interest; however, it is believed that local breast tissue levels of hormones are much more relevant than blood levels.\textsuperscript{174} While circulating steroid levels have been investigated extensively, reports on tissue steroid levels are limited because of methodological difficulties. Traditionally, scientists and clinicians have not been able to routinely measure breast tissue levels because of
inadequate technology and access to samples. Existing techniques for quantifying estrogen and other hormones in clinical samples are poorly suited for routine screening; for example, they are invasive, extracting hundreds of milligrams of tissue with a great risk of scarring. Larger samples necessitate extensive and prolonged pre-quantification processing steps, which include manual and labor-intensive processes. Thus, in the current study, we quantified breast hormone levels in breast tissue samples collected from twelve women prior to and following bilateral salpingo-oophorectomy. Measurements were made using method described in Chapter 2, that relies on digital microfluidics (DMF) and only requires samples as small as ~ 5 mg of tissue. We utilized DMF to quantify four steroids: 1) estradiol (E2), 2) androstenedione (AD), 3) testosterone (TS) and 4) progesterone (PG) from paired core needle breast biopsy (CNB) before and after oophorectomy.

3.2. Experimental

3.2.1. Recruitment of Study Subjects

Women electing to undergo prophylactic bilateral salpingo-oophorectomy (BSO) at the Women’s College Hospital, St. Michael’s Hospital or Mt. Sinai Hospital in Toronto, ON between August 2013 and April 2015 were invited to participate. Eligible subjects were approached by a study coordinator either by telephone or email after their consultation appointment with the gynaecologic oncologist. The coordinator described the study in detail and answered any questions/concerns the subject had, and acquired information regarding eligibility criteria and date and location of surgery. Subjects included women residing in the greater Toronto area that had at least one breast intact and unaffected by breast cancer at the time of the BSO, and were willing to provide blood samples and a core needle biopsy (CNB) of each intact breast before and after surgery. The study protocol was approved by the Research Ethics Board at the each recruiting hospital.
3.2.2. Data and Biological Sample Collection

Two appointments were scheduled for subjects to provide study details and samples. The first appointment was scheduled immediately before each study subject’s surgery, in which the subjects provided written consent and completed the research questionnaire to collect information on reproductive history, medication use (i.e., exogenous hormones), lifestyle factors, prophylactic mastectomy and personal cancer history. To account for the cyclic variability of hormones among premenopausal women, the first day of the last period was recorded. The pre-operative blood and breast tissue samples were collected in the operating room after the subjects had been anesthetized and prepared for surgery. The second appointment for the post-operative blood and breast tissue samples was scheduled at least four weeks after surgery, during the regularly scheduled follow-up appointment with the gynecologic oncologist. To minimize pain and discomfort to the subjects during the second appointment, local freezing was applied to the area of the CNB. Blood samples were collected into one EDTA tube (~5 mL) and one red top tube with no additives (~5 mL) by venipuncture. All samples were delivered within 30 minutes to Women’s College Hospital for processing and storage.

Breast tissue biopsy collection was performed by a trained medical fellow. Samples were obtained from the lower outer quadrants of the breast, at the same position before and after surgery in each patient. Skin was cleansed with Betadine®. A 16 gauge QuickCore (Cook Medical, Stouffville, ON) needle was placed through the skin and 3 cm into the breast tissue. The throw was extended and a 20 mm core biopsy obtained. Breast tissue samples were placed in a 2.0 mL Eppendorf tube, labelled and frozen at -80°C. Prior to analysis, each specimen was thawed, transferred to a fresh microcentrifuge tube, weighed, and homogenized by manual grinding with a disposable polypropylene pestle (VWR, ON, Canada) while kept on dry ice. Samples were further sorted on dry ice until analysis.
3.2.3 Reagents and Materials

Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON), including 1,3,5-estratriene-3,17β-diol (estradiol, E2) and 1,3,5-Estratriene-3,17β-diol-16,16,17-d3 (estradiol-d3, E2d3). 4-androsten-3,17-dione (androstenedione, AD), 4-androsten-3,17-dione-2,2,4,6,6,16,16-d7 (deuteroandrostenedione-d7, ADd7), 4-androsten-3-17β-ol-3-one (testosterone, TS), 4-androsten-3-17β-ol-3-one-16,16,17-d3 (deuterotestosterone-d3, TSD3), 4-pregnen-3,20-dione, (progesterone, PG) and 4-pregnen-3,20-dione-2,2,4,6,6,17α,21,21,21-d9 (deuteroprogesterone-d9, PGd9) were purchased from Steraloids (Newport, RI). Parylene-C dimer was from Specialty Systems (Indianapolis, IN), and Teflon-AF was obtained from DuPont (Wilmington, DE). All solvents were HPLC grade, and all other chemicals used were of the highest grade available.

Two stock solution mixtures were prepared in methanol: mixture L contained 10 µg/mL of each of the four standard "light" hormones (E2, AD, TS and PG) in neat methanol, while mixture H contained 10 µg/mL of each of the four deuterated "heavy" standard hormones (E2d3, ADd7, TSD3 and PGd9) in neat methanol and were stored at -20 °C until used. Two working solution mixtures were prepared from the stock solution mixtures, mixture L2 contained 100 ng/mL of each of the four standard light hormones (Dansylated E2, AD, TS and PG), while mixture H2 contained 100 ng/mL of each of the four standard heavy hormones (Danyslated E2d3, ADd7, TSD3 and PGd9). Each working solution was prepared as follows, briefly a 10 µL of mixture L or mixture H was pipetted into a microcentrifuge tube and allowed to evaporate until dryness at room temperature for ~10 minutes. The dried sample was then reconstituted in a 1000 µL aliquot of a 1:1 mixture of 1 mg/mL dansyl chloride in acetone, and 100 mM sodium bicarbonate (pH 10.7). The microcentrifuge tube cap was closed and it was transferred to a water bath and incubated at 60 °C for 5 min.
3.2.4. DMF Device Fabrication, Assembly, and Operation

Devices were fabricated, assembled and operated under procedures similar to those described in Chapter 2 (section 2.2.2).

3.2.5. Porous Polymer Monolith Formation

Porous polymer monolith discs were prepared under procedures similar to those described in Chapter 2 (section 2.2.3).

3.2.6. On-Chip Sample Processing

For each experiment, a tissue homogenate sample and a PPM disc were positioned on a DMF bottom plate of a device. 25 µL of lysing solvent consisting of a mixture of Dichloromethane: Acetone 80:20 was pipetted onto the tissue homogenate and allowed to dry (~30 s); the device was then assembled with a top plate. 25 µL of a dilution of mixture H (E2d3, ADd7, TSd3, PG and PGd9, 0.5 ng/mL ea.) was loaded into a solvent reservoir and driven by DMF forces onto the tissue sample. The droplet was dragged across the tissue sample in a clock-wise circular motion for 5 min, incubated statically for 10 min, and the sample-extract droplet was driven away from the tissue sample and delivered to the PPM disc (which has been activated prior to this step by dispensing a 10 µL droplet of methanol, and delivering it to the disc and incubating for 5 min, before driving the remaining methanol to waste) for clean-up. The sample-extract droplet was actuated back and forth across the PPM disc for 2 min before it was driven away to a collection reservoir.

The process described above was repeated four times, generating four separate extract-droplets, which were pooled and allowed to dry at room temperature for ~30 min. The top plate was removed, and the dried samples were reconstituted in a 25 µL aliquot of a 1:1 mixture of 1 mg/mL dansyl chloride in acetone, and 100 mM sodium bicarbonate (pH 10.7). This solution was incubated in a 60 °C water bath for 5 min, mixed with 25 µL of 50% methanol and then transferred into a 96-well plate for analysis.
3.2.7. HPLC-MS/MS

An API4000 triple stage quadrupole mass spectrometer (ABSciex, Foster City, CA, USA) was operated in multiple reaction monitoring (MRM) mode using Analyst software. Electrospray ionization (ESI) was used in positive ion mode, with ion spray voltage 5.5 kV, entrance potential 10 V and source temperature 50°C. Retention time, MRM transitions, declustering potentials, collision energies and collision cell exit potential for each analyte are listed in Table 3.1.

High performance chromatographic separations were performed using an Agilent Technologies 1100 series HPLC system (Santa Clara, California), with a CTC Analytics Leap HTS PAL Autosampler (Alexandria, Virginia). The column was an Agilent Zorbax Eclipse Plus C18 column (2.1 mm i.d. x 100 mm long, 1.8 µm particle dia.) protected by a C18 Zorbax guard column (2.1 mm i.d. x 12.5 mm long, 5 µm particle dia., Agilent) and an in-line filter (2.1 mm dia., 0.2 µm pore dia., Agilent). The HPLC was operated at ambient temperature in gradient elution mode at a flow rate of 0.1 mL/min. The gradient started with 50% mobile phase A (0.1% formic acid in DI water) and 50% mobile phase B (0.1% formic acid in methanol), changed linearly to 80% mobile phase B over 2 min, and then changed linearly again to 100% mobile phase B over the next 2 min. Mobile phase B was then held at 100% for 5 min before linearly decreasing back to 50% over 2 min and holding at 50% for 11 min for a total runtime of 22 min.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time window (min)</th>
<th>MRM transition</th>
<th>Declustering potential (V)</th>
<th>Collision energy (eV)</th>
<th>Collision cell exit potential (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>8.5-10.2</td>
<td>287/97</td>
<td>76</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>287/109</td>
<td>76</td>
<td>35</td>
<td>10</td>
</tr>
<tr>
<td>ADd7</td>
<td>8.5-10.2</td>
<td>294.9/100</td>
<td>101</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>294.9/113</td>
<td>101</td>
<td>37</td>
<td>8</td>
</tr>
<tr>
<td>TS</td>
<td>8.9-10.3</td>
<td>289/97</td>
<td>86</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>289/109</td>
<td>86</td>
<td>35</td>
<td>8</td>
</tr>
<tr>
<td>TSd3</td>
<td>8.9-10.3</td>
<td>292/97</td>
<td>61</td>
<td>37</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>292/109</td>
<td>61</td>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>PG</td>
<td>9.75-11.25</td>
<td>315/97</td>
<td>96</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>315/109</td>
<td>96</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td>PGd9</td>
<td>9.75-11.25</td>
<td>324/100</td>
<td>106</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>324/113</td>
<td>106</td>
<td>37</td>
<td>10</td>
</tr>
<tr>
<td>Dansylated E2</td>
<td>12.2-13.6</td>
<td>506/171</td>
<td>45</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>Dansylated E2d3</td>
<td>12.2-13.6</td>
<td>509/171</td>
<td>45</td>
<td>35</td>
<td>6</td>
</tr>
</tbody>
</table>
3.2.8. Quantitative Analysis

External calibration curves were constructed by measuring seven different concentrations of standards from dilutions of solution mixture L2 (0, 20, 50, 100, 500, 2000 and 5000 pg/mL ea.) formed in 50:50 methanol:DI water. Each standard also contained 1000 pg/mL of deuterated internal standards. The ratios of the area under the curve (AUC) for each MRM chromatogram for each non-deuterated steroid product ion relative to that of its corresponding deuterated internal standard was plotted as a function of concentration. Steroid concentrations in tissue samples were determined from an averaged regression line equation generated from two calibration plots, collected before and after each tissue sample measurement. Each tissue hormone-level was expressed as a ratio of absolute mass of analyte to tissue weight, values were corrected for extraction efficiency when possible (described in Chapter 2).

3.2.9. Immunoassay

Given the limited amount of serum sample available, only E2 and PG were quantified by immunoassays in serum samples by staff at the LifeQuest Centre for Reproductive Medicine. Each concentration (pM or nM) was rescaled as analyte mass/serum mass, using previously reported serum density value of 1.024 g/mL.\textsuperscript{177}

3.2.10. Statistical Analysis

The mean hormone concentrations in breast tissue were determined prior to and following surgery for each participant. For those women with CNB samples obtained from both breasts, the average of all the samples was included. The percent (%) change in hormone levels following surgery was estimated by calculating the ratio of the difference between the pre-BSO and post-BSO levels to the pre-BSO level.

3.3. Results

A total of 29 women were eligible for the current research study and were invited to participate. Fourteen women consented to participate including three women with a \textit{BRCA1} mutation, nine women with a \textit{BRCA2} mutation, and two \textit{BRCA} negative women.
Two women were excluded from the final analysis; both were BRCA2 mutation carriers, both patients declined the second CNB because of the development of a hematoma at the site of the first CNB. A total of 12 women completed the study; three BRCA1 mutation carriers, seven BRCA2 mutation carriers, and two BRCA negative women.

The characteristics of the 12 women included in the study are summarized in Table 3.2. Seven of the 12 BRCA mutation carriers were premenopausal and five were postmenopausal at the time of enrolment. Both non-carriers were postmenopausal at the time of enrolment into the study. All participating women provided samples from both breasts, and two women with previous history of breast cancer provided a sample from the one unaffected breast only. One of the BRCA negative women was using tamoxifen at the time of enrolment (and after surgery) because of a previous breast cancer diagnosis. Three of the women that were premenopausal at the time of enrolment initiated HRT immediately after surgery.

Table 3.2. Characteristics of women at the time of bilateral salpingo-oophorectomy, by BRCA mutation status.

<table>
<thead>
<tr>
<th>BRCA1 mutation carrier (n=3)</th>
<th>BRCA2 mutation carrier (n=7)</th>
<th>Non-carrier (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean years (range)</td>
<td>45.5 (38.2 – 53.2)</td>
<td>48.7 (42.5 – 58.4)</td>
</tr>
<tr>
<td>Follow-up, mean months (range)</td>
<td>26.8 (3.1 – 73.1)</td>
<td>5.9 (2.0 – 11.0)</td>
</tr>
<tr>
<td>Postmenopausal status, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>1 (33%)</td>
<td>2 (29%)</td>
</tr>
<tr>
<td>Premenopausal</td>
<td>2 (67%)</td>
<td>5 (71%)</td>
</tr>
<tr>
<td>Prior breast cancer diagnosis, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1 (33%)</td>
<td>2 (29%)</td>
</tr>
<tr>
<td>No</td>
<td>2 (67%)</td>
<td>5 (71%)</td>
</tr>
<tr>
<td>Tamoxifen, n (%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Core needle biopsy collection, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left breast only</td>
<td>1 (33%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Right breast only</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Both breasts</td>
<td>2 (67%)</td>
<td>7 (100%)</td>
</tr>
</tbody>
</table>
Table 3.3 and Figures 3.1-3.8 summarize the mean hormone concentrations (E2, AD, TS and PG) in the CNB samples obtained from the breast tissue of the women prior to and after surgery, as well as the percent change in hormone levels, by menopausal status. Among premenopausal women who did not initiate HRT immediately after surgery (Study ID 1-4), there was a reduction in breast E2 (range 33-94%) for all four women. Breast E2 levels remained similar or higher among the women in Group 2 (Study ID 5-7), all of which were premenopausal prior to surgery and initiated HRT (a combined regimen of premarin and progesterone) immediately following surgery. There was either a reduction or no change in breast tissue TS levels for all subjects, except for one that had a large increase (Study ID 3). There was mostly reduction or no change in AD levels, with only one patient showing significant increase (Study ID 5). The effect of surgery on breast tissue levels of PG was more variable.

Among postmenopausal women not on tamoxifen (Group 3), there was a reduction in breast E2 among three of the four women (25-70%). The breast E2 levels for Study ID 10 were low. There was one postmenopausal woman who remained on tamoxifen following surgery and her breast E2 level decreased by 44% following surgery (Study ID 12). There was a reduction in breast TS levels among all five postmenopausal women. The effect of surgery on breast tissue breast AD levels varied. Breast PG levels were mostly very low (or not detectable) among the postmenopausal women (Groups 3 and 4). In summary, a over 50% of patients exhibited reduction in breast tissue E2, AD and TS levels post-surgery, while breast PG levels were variable but mostly non-detectable.

The corresponding serum E2 and PG levels of the study participants prior to and following surgery are summarized in Table 3.4. Among premenopausal women (Study IDs 1-7), there was a reduction in serum E2 levels among five of the seven women (range 7.4-79%), while serum E2 levels were not measured in Study ID 1 and ID 5. The post-surgery serum E2 levels of the premenopausal women were similar to those of the pre- and post-surgery levels of the postmenopausal subjects. Serum E2 levels were low in the postmenopausal group (Study IDs 8-12) and generally remained unaffected with surgery. Pre- and post-surgery serum PG levels were relatively similar among the premenopausal
and postmenopausal subjects. Some, but not all, serum PG levels decreased following surgery.

Table 3.3. Hormone levels in core needle biopsy (CNB) of breast tissue samples among the study participants (normalized by sample mass) before and after surgery. Raw values are the measured amounts; corrected values are extrapolated from the extraction efficiencies.

<table>
<thead>
<tr>
<th>ID</th>
<th>E2 (fmol/mg)</th>
<th>AD (fmol/mg)</th>
<th>TS (fmol/mg)</th>
<th>PG (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corrected</td>
<td>Corrected</td>
<td>Corrected</td>
<td>Corrected</td>
</tr>
<tr>
<td>1</td>
<td>0.7</td>
<td>0.1</td>
<td>85.7</td>
<td>8.3</td>
</tr>
<tr>
<td>2</td>
<td>0.6</td>
<td>0.4</td>
<td>33.3</td>
<td>7.7</td>
</tr>
<tr>
<td>3</td>
<td>13.5</td>
<td>0.8</td>
<td>94.1</td>
<td>7.6</td>
</tr>
<tr>
<td>4</td>
<td>6.4</td>
<td>1.2</td>
<td>81.3</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>5.4</td>
<td>6.8</td>
<td>-25.9</td>
<td>1.6</td>
</tr>
<tr>
<td>6</td>
<td>0.2</td>
<td>0.7</td>
<td>-250.0</td>
<td>1.2</td>
</tr>
<tr>
<td>7</td>
<td>1.6</td>
<td>4.0</td>
<td>-150.0</td>
<td>2.7*10^1</td>
</tr>
<tr>
<td>8</td>
<td>0.4</td>
<td>0.3</td>
<td>25</td>
<td>2.0</td>
</tr>
<tr>
<td>9</td>
<td>0.9</td>
<td>0.6</td>
<td>33.3</td>
<td>2.6</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>0.4</td>
<td>-</td>
<td>1.4</td>
</tr>
<tr>
<td>11</td>
<td>8.6*10^1</td>
<td>2.6*10^1</td>
<td>70.3</td>
<td>2.9</td>
</tr>
<tr>
<td>12</td>
<td>1.3*10^2</td>
<td>7.0*10^1</td>
<td>44.1</td>
<td>4.0</td>
</tr>
</tbody>
</table>

ND = not detectable
- : % reduction was not calculated for women with ND levels

The subjects were categorized into four groups: Group 1: premenopausal (Study ID: 1-4), Group 2: premenopausal on HRT (Study ID: 5-7), Group 3: postmenopausal not on any medication (Study ID: 8-11), and Group 4: postmenopausal on tamoxifen (Study ID: 12). DMF method precision (based on data obtained from processing rat fat tissue samples) was 22.5, 21.4 and 11.2% RSD for E2, AD and TS respectively.
<table>
<thead>
<tr>
<th>Study ID</th>
<th>E2 (fmol/mg)</th>
<th>PG (fmol/mg)</th>
<th>% Reduction</th>
<th>Study ID</th>
<th>E2 (fmol/mg)</th>
<th>PG (fmol/mg)</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>% Reduction</td>
<td>Before</td>
<td>After</td>
<td>% Reduction</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>1.4*10⁻¹</td>
<td>-</td>
<td>9.8</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.4*10⁻¹</td>
<td>1.2*10⁻¹</td>
<td>52.0</td>
<td>2.9</td>
<td>3.7</td>
<td>-25.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.6*10⁻¹</td>
<td>2.4*10⁻¹</td>
<td>7.4</td>
<td>6.1</td>
<td>2.7</td>
<td>55.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.6*10⁻¹</td>
<td>1.2*10⁻¹</td>
<td>78.9</td>
<td>3.5</td>
<td>3.6</td>
<td>-2.8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.2*10⁻¹</td>
<td>1.9*10⁻¹</td>
<td>12.6</td>
<td>2.9</td>
<td>3.7</td>
<td>-26.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5.2*10⁻¹</td>
<td>1.8*10⁻¹</td>
<td>66.0</td>
<td>5.0*10¹</td>
<td>1.2*10¹</td>
<td>76.6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.1*10⁻¹</td>
<td>1.5*10⁻¹</td>
<td>-37.0</td>
<td>4.3</td>
<td>3.3</td>
<td>24.0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.6*10⁻¹</td>
<td>1.4*10⁻¹</td>
<td>11.5</td>
<td>4.4</td>
<td>3.6</td>
<td>18.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>1.3*10⁻¹</td>
<td>-</td>
<td>-</td>
<td>3.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.3*10⁻¹</td>
<td>1.2*10⁻¹</td>
<td>4.8</td>
<td>6.7</td>
<td>2.9</td>
<td>56.5</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.5*10⁻¹</td>
<td>2.1*10⁻¹</td>
<td>-38.6</td>
<td>4.2</td>
<td>4.9</td>
<td>-17.1</td>
<td></td>
</tr>
</tbody>
</table>

- : % reduction was not calculated as serum values were not measured.

The subjects were categorized into four groups: Group 1: premenopausal (Study ID: 1-4), Group 2: premenopausal on HRT (Study ID: 5-7), Group 3: postmenopausal not on HRT (Study ID: 8-11), and Group 4: postmenopausal on tamoxifen (Study ID: 12).
Figure 3.1. Tissue (solid lines, left axis) and serum (dashed lines, right axis) estradiol levels in seven premenopausal women prior to and following bilateral salpingo-oophorectomy. The patients represented in plots E-G went on hormone replacement therapy immediately after surgery. In plots marked with an asterisk (*) serum values were not measured.
Figure 3.2. Tissue (solid lines, left axis) and serum (dashed lines, right axis) estradiol levels in five postmenopausal women prior to and following bilateral salpingo-oophorectomy. The patient represented in plot E was on the aromatase-inhibitor, tamoxifen. In plots marked with an asterisk (*), serum values were not measured.
Figure 3.3. Tissue androstendione levels (solid lines) in seven *premenopausal* women prior to and following bilateral salpingo-oophorectomy. The patients represented in plots E-G went on hormone replacement therapy immediately after surgery.
Figure 3.4. Tissue androstendione levels (solid lines) in five *postmenopausal* women prior to and following bilateral salpingo-oophorectomy. The patient represented in plot E was on the aromatase-inhibitor, tamoxifen.
Figure 3.5. Tissue testosterone levels (solid lines) in five premenopausal women prior to and following bilateral salpingo-oophorectomy. The patients represented in plots D-E went on hormone replacement therapy immediately after surgery. Note that two additional premenopausal patients (not shown) had non-detectable TS levels.
Figure 3.6. Tissue testosterone levels (solid lines) in five postmenopausal women prior to and following bilateral salpingo-oophorectomy. The patient represented in plot E was on the aromatase-inhibitor, tamoxifen.
Figure 3.7. Tissue (solid lines, left axis) and serum (dashed lines, right axis) progesterone levels in seven premenopausal women prior to and following bilateral salpingo-oophorectomy. The patients represented in plots E-G went on hormone replacement therapy immediately after surgery. In plots marked with an asterisk (*), serum values were not measured.
Figure 3.8. Tissue (solid lines, left axis) and serum (dashed lines, right axis) progesterone levels in postmenopausal women prior to and following bilateral salpingo-oophorectomy. The patient represented in plot E was on the aromatase-inhibitor, tamoxifen. In plots marked with an asterisk (*), serum values were not measured.
3.4. Discussion

There is substantial evidence supporting an increased risk of breast cancer with high circulating estrogen and androgen levels among both pre- and post-menopausal women in the general population.\(^{165}\) Further, it is known that oophorectomy is associated with a reduction in breast cancer risk, believed to be related to the reduction in circulating ovarian hormone levels.\(^{130,178}\) The relationship between oophorectomy and local levels of steroid hormones in breast tissue has never before been explored; we sought to remedy this deficit here.

The data generated using the new technique are reported in Figures 3.1-3.8 and in Table 3.3, as shown, there is a general trend for reduction in E2, AD and TS levels in the breast tissue after oophorectomy, with some outliers (e.g., the patients who went on HRT showed increases in breast E2, as in Fig. 3.1 E-G, etc.). The trend for PG is less clear, as is the relationship between tissue levels and serum levels (Table 3.4) within individuals.

In summary, this represents the first study to quantify sex hormone levels in CNB in patients prior and post to oophorectomy. A key goal in our work here was to establish initial report of tissue hormone levels in pre- and post-menopausal females and its variation with oophorectomy, a second goal was to introduce a method that can be easily adopted in the future for routine analysis of tissue hormone levels. Using DMF we were able to achieve the first goal, and we have successfully reported tissue hormone concentration levels in females prior and post oophorectomy surgery, our results suggest that oophorectomy has led to a consistent decrease in E2, AD and TS tissue levels in the > 50% of patients.

Tables 3.5 and 3.6 summarize data from a few earlier studies similarly investigating breast tissue hormone levels in pre- and post-menopausal women, respectively\(^{179-182}\). We were not able to locate any reports of tissue E2, AD, TS or PG levels post-oophorectomy, however, tissue E2 levels in pre- and post-menopausal females have been quantified by a few number of studies. There has been marked variation in the breast tissue E2 concentrations in pre- and post-menopausal females reported across the different studies. Studies investigating tissue E2 levels in premenopausal females have reported, median E2 concentration of 0.48 fmol/mg (Thijssen et. al\(^{180}\)), a mean concentration of 5 fmol/mg
(Cortés-Gallegos et. al.\textsuperscript{183}) and a range of 0.18 - 1.8 fmol/mg protein (Van Landeghem et. al.\textsuperscript{182}). Our data for the levels of E2 in premenopausal pre-surgical E2 levels fall close to the values obtained by previous reports.

Data on postmenopausal tissue E2 levels were very limited, most studies report only mean or median E2 values (Table 3.6).\textsuperscript{180,184,185}, the same thing applies to AD, TS and PG, previous reports on their endogenous concentrations were very limited and there was no established biological range for tissue concentrations of these hormones in pre- or post-menopausal females.

In addition, all of the reported studies have employed very invasive surgical sampling methods to collect hundreds of milligrams of tissue, such procedures are not suitable for routine testing, most samples were collected from patients undergoing mastectomy. Due to larger sample size (hundreds of milligrams of tissue), extensive tissue preparation steps have been employed, and radioimmunoassay (RIA) was used to quantify extracted hormones, RIA have been reported to be a powerful technique with exquisite sensitivity, however, preliminary tissue purification and separation steps are required to avoid cross reactivity which may lead to false positive results, RIA also has limited multiplexing capabilities.

\textbf{Table 3.5. Summary of previously published hormone concentrations in non-malignant breast tissue samples from premenopausal human subjects normalized by sample}

<table>
<thead>
<tr>
<th></th>
<th>E2</th>
<th>AD</th>
<th>TS</th>
<th>PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thijssen et. al.\textsuperscript{180} (median, fmol/mg)</td>
<td>0.48</td>
<td>0.78</td>
<td>1.03</td>
<td>-</td>
</tr>
<tr>
<td>Cortés-Gallegos et. al.\textsuperscript{185} (Mean ±SD, fmol/mg)</td>
<td>5 ± 1.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Van Landeghem et. al.\textsuperscript{186} (Range, fmol/mg)</td>
<td>0.18 - 1.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3.6. Summary of previously published hormone concentrations in non-malignant breast tissue samples from postmenopausal human subjects normalized by sample

<table>
<thead>
<tr>
<th></th>
<th>E2</th>
<th>AD</th>
<th>TS</th>
<th>PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vermulen et. al.</td>
<td>2.9 - 55</td>
<td>6.3<em>10^4 - 1.4</em>10^3</td>
<td>1.0-1.5*10^3</td>
<td>1.3<em>10^2 - 1.5</em>10^3</td>
</tr>
<tr>
<td>(range, fmol/mg protein)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thijssen et. al</td>
<td>0.19 &amp; 0.32</td>
<td>0.58</td>
<td>0.37</td>
<td>-</td>
</tr>
<tr>
<td>(median, fmol/mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bonney et. al.</td>
<td>0.6 ± 0.18</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Mean ±SD, fmol/mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chetrite et. al.</td>
<td>0.6 ± 0.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Mean ±SD, fmol/mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* steroid concentration was normalized tissue protein contents.

In planning this study, we originally envisioned much larger sample sizes; unfortunately, with the small samples tested here, strong conclusions cannot be drawn. We can conclude, however, that there may be some merit in conducting larger studies in the future, particularly if patient histories can be followed to determine clinical outcomes. We propose that the DMF tissue processing platform will encourage future research into this area that would allow for design of high-powered studies to establish a correlation between breast cancer risk and tissue hormone concentration.
Chapter 4
Towards A Personalized Approach To Aromatase Inhibitor Therapy: A Digital Microfluidic Platform For Rapid Analysis Of Estradiol In Core-Needle-Biopsies

Despite the advances in breast cancer prevention and treatment, the variability has revealed the need for a more “personalized” approach to medicine, in which treatments are tailored to each patient’s biology. Motivated by this idea, we introduce a technique that allows for quantification of small-molecule analytes directly from core needle biopsy (CNB) samples on a miniaturized platform. The new technique powered by digital microfluidics (DMF), integrates tissue liquid extraction and magnetic bead-based competitive immunoassay for quantification of estradiol (E2) in mg-sized CNB samples. Each measurement (from start to finish) requires ~40 minutes, a duration consistent with a visit to a doctor’s office. The performance of the technique was validated by gold-standard HPLC-MS/MS analysis, and was applied to evaluate human patient samples before and after a course of treatment with aromatase inhibitor therapy. Here we propose that the new technique has great potential for eventual use as a fast, automated, and inexpensive instrument for the quantitative analysis of small molecules for on-site decision making towards a personalized medicine approach.

4.1. Introduction

The classical approach to treating cancer and other systemic illnesses employs a “standard of care” regime, in which all patients receive similar interventions. Although the efficacy of treatments has improved over the decades, the variability in outcomes has revealed the need for a “personalized” approach to treatment, in which therapies are tailored to each patient’s specific response. This has the potential to maximize the impact of therapeutic measures and minimize adverse effects caused by unnecessary intervention. A key barrier to realizing the potential benefits of personalized medicine is a lack of rapid, accessible diagnostic tools that can be used in the clinician’s office.
Motivated by this need, this study introduces a diagnostic tool that may prove useful for personalized treatment of estrogen receptor (ER)-positive breast cancer. The traditional approach to treating this disease is the prescription of selective estrogen receptor (ER) modulators (SERMs), which inhibit ER activity in tumor tissue. Briefly, when estradiol (E2) binds ER in normal or tumor cells, a biochemical cascade is initiated that eventually activates transcription of proteins responsible for cell proliferation.\textsuperscript{188} SERMs are thus used to inhibit this effect, which limits tumor cell proliferation. More recently, an alternative treatment known as aromatase inhibitor therapy (AIT) has become popular for the treatment of post-menopausal ER-positive breast cancer patients. AIT, which acts by inhibiting the biosynthesis of E2, is believed to be more effective than SERMs in some patients because of the reported partial agonistic activity of SERMs at the ER.\textsuperscript{189} In addition, there is a number of catechol-estrogens and other estrogen metabolites that are believed to be mutagenic; the formation of these metabolites is inhibited by AIT but not by SERMs.\textsuperscript{190} Further, there is evidence of cross-talk between the steroid receptor pathway and growth-factor-receptors (co-expressed by breast cancer carcinomas) that may lead to SERM-resistance.\textsuperscript{191} Finally, postmenopausal women with early or advanced ER-positive breast cancer have reported fewer side-effects for AIT relative to SERMs.\textsuperscript{192}

In addition to the recent emergence of AIT as a front-line treatment for post-menopausal patients with ER-positive breast cancer, AIT has also long been the standard-of-care adjuvant therapy prescribed to healthy patients that were previously diagnosed with contralateral breast cancer.\textsuperscript{193} While this treatment can avert the development of 70-80\% of ER-positive breast cancers,\textsuperscript{194} AIT has little effect on outcome for the remainder of patients, likely caused by inter-individual variations in aromatase activity.\textsuperscript{195} Patient-specific variations in drug resistance are also likely contributors to this effect, which include intrinsic drug resistance (for tumors that are inherently non-responsive to endocrine treatment), and acquired resistance, in which tumors initially respond to the endocrine agent, but then become resistant during the course of treatment. In the latter case, the resistance is often agent-selective, and can be overcome by changing the type of AIT that is prescribed.\textsuperscript{196,197}
Given the variability in patient response to AIT, we propose that regular monitoring of E2 levels in breast tissue during the course of treatment would be useful to determine the effectiveness of the therapy. As a step towards personalized treatment, in Chapter 2 we have reported a digital microfluidic strategy for quantifying E2 and other hormones in core needle biopsy (CNB) samples of breast tissue. CNBs are ideal for personalized medicine, as the ~milligram-sized samples can be collected in the doctor’s office, without general anaesthesia or risk of scarring. But our original technique relied on analysis by liquid chromatography and tandem mass spectrometry (LC-MS/MS); this type of instrument is not readily available in clinics or small labs, making it inappropriate for the rapid turn-around needed to guide personalized care.

Here we report a technique for the rapid determination of patient response to AIT: an integrated, portable microfluidic platform that allows quantification of E2 in CNB samples. The instrument is small (shoe-box size) and can be easily operated outside of the laboratory, making it potentially useful for on-site decision-making. The new technique, powered by digital microfluidics, integrates tissue-liquid extraction and a magnetic bead-based competitive immunoassay in a miniaturized format, facilitating the quantification of estradiol from milligram-sized CNB samples. We propose that this system may eventually be useful in aiding physicians as they select and dose aromatase inhibitors in the management of breast cancer (Figure 4.1). If successful, this system will join the rising tide of microfluidic techniques that are paving the way for a personalized medicine approach to healthcare.198-200
Figure 4.1. A personalized regime for aromatase inhibitor therapy (AIT). (A) The patient is welcomed and prepared for sample collection. (B) A core needle biopsy (CNB) sample is collected. (C) CNB estradiol (E2) is measured using a custom digital microfluidic instrument for CNB processing and analysis (photo). (D) Personalized AIT is prescribed.

4.2. Experimental

4.2.1. Reagents and Materials

Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON), including 1,3,5-Estratriene-3,17β-diol (estradiol, E2) and 1,3,5-Estratriene-3,17β-diol-16,16,17- d3 (estradiol-d3, E2d3). Deionized (DI) water had a resistivity of 18 MΩ·cm at 25°C. Stock solutions (1 mg/mL each) of E2 and E2d3 were prepared in neat methanol, and working solutions of each standard (10 µg/mL each) were formed in methanol by serial dilution. All stock and working solutions were stored at -20°C until use. Standard solutions (100 ng/mL each) of dansylated E2 and dansylated E2d3 were prepared by evaporating 10 µL samples of working solutions of E2 or E2d3 in microcentrifuge tubes to dryness at room temperature for ~10 min, and then reconstituting in 1000 µL of a 1:1 mixture of 1 mg/mL dansyl chloride in acetone and
aqueous 100 mM sodium bicarbonate (pH 10.7). In each case, the cap was affixed and the tube was transferred to a water bath and incubated at 60°C for 5 min.

Rabbit monoclonal anti-E2 coated paramagnetic microparticles, and E2 assay diluent (containing surfactant in citrate buffer) were adapted from ARCHITECT immunoanalyzer reagent kits obtained from Abbott Laboratories (Abbott Park, IL). Estradiol conjugated with horse radish peroxidase (E2-HRP, conjugated via 6-CMO), was purchased from BiosPacific (Emeryville, CA). SuperSignal ELISA Femto chemiluminescent substrate, comprising separate solutions of stabilized hydrogen peroxide (H₂O₂) and luminol/enhancer, was purchased from Thermo Fisher Scientific (Rockford, IL). SuperBlock™ (a proprietary mixture of proteins in phosphate buffered saline), used as both particle and conjugate diluent, was purchased from Thermo Fisher Scientific. Prior to use, all reagents were supplemented with Tetronic 90R4 (0.1 % v/v) obtained from Sigma Chemical (Oakville, ON). A DMF-compatible Tris wash buffer was formed from Tris-base (0.35 g/L), Tris-HCl (1.10 g/L), NaCl (8.367 g/L), and Tetronic 90R4 (0.1 % v/v), an immunoassay reconstitution buffer was formed from bovine serum albumin (4% w/v) in Dulbecco’s phosphate buffered saline (DPBS) with Tetronic 90R4 (0.1% v/v), and LC-MS/MS reconstitution buffer was formed from a 1:1 mixture of 1 mg/mL dansyl chloride in acetone and aqueous 100 mM sodium bicarbonate (pH 10.7).

4.2.2. DMF Device Fabrication, Assembly, and Operation

DMF devices were formed as described in Chapter 2. Unique to this work, each top-plate was patterned to include one 3 mm-diameter circular region of exposed ITO (known as the “hydrophilic anchor”), formed by lift-off of a circular piece of dicing tape (applied and removed before and after spin-coating). Devices were assembled by joining a top and bottom plate with a spacer formed from two pieces of 3M Scotch double-sided tape (St. Paul, MN) with a total spacer thickness of 180 μm. Unit droplets (covering one actuation electrode) in this system were thus ~0.8 μL.

Droplet movement, extraction, reaction, and detection was implemented using an integrated, home-made instrument²⁰¹ that allows for control of droplet position, control of a magnet mounted on a step-motor that can be moved to enable separation of magnetic
particles, as well as a photomultiplier tube (PMT) to measure chemiluminescence. Aliquots of reagents were loaded onto a DMF device by pipetting a droplet onto the bottom plate at the edge of the top plate, and simultaneously applying sine wave voltages (~100 $V_{rms}$, 10 kHz) between the top plate electrode and successive electrodes on the bottom plate via a custom pogo-pin connector, to draw the fluid into the reservoir and further manipulate droplet movement. The automation system (including the magnet) was programmed and managed by Microdrop, an open-source application for the manipulation of droplets on DMF devices.\textsuperscript{127}

4.2.3. On-Chip Tissue Extraction

E2 was extracted from tissue samples on DMF devices in a six-step procedure. (1) A tissue homogenate (preparation described in sections 4.2.6-4.2.7) was collected from a microcentrifuge tube and then loaded onto the bottom plate of a device. A top plate was affixed onto the device, and 20 µL extraction solvent (neat methanol for immunoassays or 10 ng/mL E2d3 in methanol for LC-MS/MS experiments) was loaded into a reservoir. (2) A 4.8 µL droplet of extraction solvent was dispensed onto the array and delivered to the CNB sample, and E2 was extracted into the droplet by moving in a circular pattern around the tissue sample for 5 min. (3) The sample-extract droplet was driven away from the tissue and delivered to the hydrophilic anchor on the top plate for solvent evaporation (5 min at room temperature). (4-5) Steps 2 and 3 were repeated. (6) The analyte dried on the hydrophilic anchor was then redissolved in an appropriate solvent for analysis. For immunoassay experiments, step (6) comprised dispensing a 4.8µL droplet of immunoassay reconstitution buffer onto the anchor and actuating the droplet for 30 s around the spot, followed by moving the droplet onto the working area of the device for on-chip immunoassay processing (see section 4.2.4). For LC-MS/MS, in step (6) the top plate was removed and a 25 µL aliquot of LC-MS/MS reconstitution buffer was pipetted onto the anchor, where it was manually agitated for 30 seconds. This solution was collected into a microcentrifuge tube, incubated in a 60 °C water bath for 5 min, mixed with 25 µL of 50% methanol/DI water and then transferred into polypropylene vials for analysis by LC-MS/MS.
4.2.4. On-Chip Immunoassay

E2 was measured in standards and in tissue extract (generated on-chip as above) in a sixteen–step digital microfluidic immunoassay. (1) A 1.6 µL droplet containing paramagnetic particles was dispensed from a reservoir and separated from the diluent by engaging the magnet. (2) A 4.8 µL droplet of E2 standard or reconstituted sample extract was delivered to the immobilized particles for resuspension. (3) The droplet was actively mixed with the particles for 6 min before engaging the magnet and driving the supernatant to waste. (4) A 1.6 µL droplet of wash-buffer was dispensed onto the array, driven to the particles, and the particles were resuspended and mixed for 10 s. The magnet was engaged and the supernatant was driven to waste. (5-7) Step (4) was repeated 3x. (8) A 1.6 µL droplet of conjugate solution was dispensed and delivered to the immobilized particles, which were resuspended. (9) The droplet was actively mixed for 2 min. (10-13) Step (4) was repeated 4x. (14) A 0.8 µL droplet of H₂O₂ was dispensed and delivered to the immobilized particles. (15) A 0.8 µL droplet of luminol/enhancer solution was dispensed and delivered to the immobilized particles, which were resuspended in the combined (H₂O₂ and luminol/enhancer) droplet. (16) The droplet was actively mixed for 10 min, and chemiluminescence was measured using the integrated H10682-110 PMT (Hamamatsu Photonics K.K., Hamamatsu, Japan).

For quantification of E2 in tissue extract, a standard calibration curve was generated, which consisted of signal measured from ten different concentrations of E2 (0, 10, 50, 100, 500, 1000, 2500, 5000, 10000 and 20000 pg/mL) which were formed by serial dilutions of E2 stock solution in assay diluent. The chemiluminescent signal was plotted as a function of concentration and fit with a four-parameter logistic curve. The chemiluminescent signals observed from each tissue extract were compared to the curve to determine the concentration measured in the sample, which was reported as a ratio of absolute amount of analyte detected relative to sample mass.
4.2.5. HPLC-MS/MS

E2 was measured from standards or tissue extract (generated on-chip, as above). 30 µL samples were loaded from 250 µL polypropylene vials (Agilent Technologies, Santa Clara, CA) positioned in a 54-vial plate via a CTC Analytics Leap HTS PAL Autosampler (Alexandria, Virginia). Chromatographic separations were performed using an Agilent Technologies 1100 series HPLC system (Santa Clara, CA), with an Agilent Zorbax Eclipse Plus C18 column (2.1 mm i.d. x 100 mm long, 1.8 µm particle dia.) protected by a C18 Zorbax guard column (2.1mm i.d. x 12.5 mm long, 5 µm particle dia., Agilent) and an in-line filter (2.1 mm dia., 0.2 µm pore dia., Agilent). The HPLC was operated at ambient temperature in gradient elution mode at a flow rate of 0.1 mL/min. The gradient started with 50% mobile phase A (0.1% formic acid in DI water) and 50% mobile phase B (0.1% formic acid in methanol), changed linearly to 80% mobile phase B over 2 min, and then changed linearly again to 100% mobile phase B over the next 2 min. Mobile phase B was then held at 100% for 5 min before decreasing linearly back to 50% over 2 min and holding at 50% for 11 min, for a total runtime of 22 min. HPLC eluent was interfaced into an API4000 triple stage quadrupole mass spectrometer (ABSciex, Foster City, CA, USA) via an electrospray ionization (ESI) source. The source voltage was 5.5 kV, the declustering potential was 45 V, the entrance potential was 10 V, the collision energy was 35 eV, the collision cell exit potential was 6 V, the source temperature was 50 ºC, and the multiple reaction monitoring (MRM) transitions of dansylated E2 and dansylated E2d3 were 506/171 and 509/171, respectively.

For quantification of E2 in tissue extract, standard calibration curves were generated, which consisted of signal measured from seven different concentrations of dansylated E2 (0, 20, 50, 100, 500, 2000 and 5000 pg/mL) containing 2000 pg/mL of dansylated E2d3 which were formed by serial dilutions in 50:50 methanol:DI water. The ratios of the areas under the curve (AUC) in MRM chromatograms for dansylated E2 product ions relative to those of dansylated E2d3 were plotted as a function of concentration and fit with a linear regression. A new calibration curve was generated before and after each set of tissue extract were analyzed, and E2 concentration in the extract was calculated from the
average regression lines from the two plots. Measured E2 was then reported as a ratio of absolute amount of analyte detected relative to sample mass.

4.2.6. Rat Tissue Samples

Rat breast tissue samples were obtained from lactating and non-lactating female rats at the animal facility in the Donnelly Centre for Cellular and Biomolecular Research. Rats were euthanized without anaesthesia using CO\(_2\) for ~4 min; ~5 mg tissue specimens were collected from the breast region with scissors and were instantly frozen under liquid nitrogen before storing at ~80°C until use. Prior to analysis, specimens were thawed, sectioned, transferred to microcentrifuge tubes, and weighed, followed by homogenization by manual grinding with a disposable polypropylene pestle (VWR, ON, Canada), while on dry ice. Samples were then kept on dry ice until analysis. In some experiments, exogenous E2 was spiked into samples prior to homogenization (as described below).

Rat tissue samples were used for three types of experiments. (1) To evaluate HPLC-MS/MS analytical performance, tissue samples from lactating rats were spiked with aliquots of E2 to give exogenous amounts of E2 as stated in Table 4.1. These samples were extracted on-chip and analyzed by HPLC-MS/MS (as above) to determine the amount of measured (or “found”) E2 in each sample. After subtracting the amount of found endogenous E2 (measured in the sample with no spiked analyte), the data was plotted against the spiked amounts, fitted with a linear regression, and the extraction efficiency was calculated from the average ratio of found/spiked E2 for the 10 spiked samples. (2) To evaluate on-chip immunoassay performance and compare it to that of HPLC-MS/MS, two sets of tissue samples from lactating rats were spiked with exogenous aliquots of E2 to give final amounts of E2 described in Table 4.2. One set of samples was measured by HPLC-MS/MS while the other set was measured by on-chip immunoassay (as above). After subtracting the amount of found endogenous E2 from both types of measurements, the found immunoassay measurements were plotted against those from the HPLC-MS/MS, and fitted with a linear regression. In addition, on-chip immunoassay extraction efficiency was calculated from the average ratio of found to
spiked E2 for the 10 spiked samples. (3) To evaluate the difference between native E2 in lactating and non-lactating rats, samples from each type of rat were measured by on-chip immunoassay. These raw values were multiplied by the reciprocal of the immunoassay extraction efficiency to determine the corrected amounts.

**Table 4.1.** Final amounts (fmol/mg) of exogenous E2 standards spiked into eleven breast tissue samples obtained from lactating rats.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>E2 amount (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td>4</td>
<td>24.0</td>
</tr>
<tr>
<td>5</td>
<td>26.1</td>
</tr>
<tr>
<td>6</td>
<td>54.8</td>
</tr>
<tr>
<td>7</td>
<td>61.2</td>
</tr>
<tr>
<td>8</td>
<td>64.0</td>
</tr>
<tr>
<td>9</td>
<td>100.1</td>
</tr>
<tr>
<td>10</td>
<td>117.8</td>
</tr>
<tr>
<td>11</td>
<td>124.5</td>
</tr>
</tbody>
</table>
Table 4.2. Final amounts (fmol/mg) of exogenous E2 standards spiked into two sets of breast tissue samples obtained from lactating rats.

<table>
<thead>
<tr>
<th>Tissue Set #</th>
<th>E2 amount (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>8.2</td>
</tr>
<tr>
<td>3</td>
<td>8.5</td>
</tr>
<tr>
<td>4</td>
<td>12.3</td>
</tr>
<tr>
<td>5</td>
<td>19.1</td>
</tr>
<tr>
<td>6</td>
<td>30.4</td>
</tr>
<tr>
<td>7</td>
<td>30.8</td>
</tr>
<tr>
<td>8</td>
<td>62.0</td>
</tr>
<tr>
<td>9</td>
<td>69.8</td>
</tr>
<tr>
<td>10</td>
<td>116.9</td>
</tr>
</tbody>
</table>

4.2.7. Human Samples

Ethical approval was granted by the Research Ethics Board of Mount Sinai Hospital (Reference number 07-0015A). Written informed consent was obtained from each participant before enrolment. Paired core needle biopsy breast tissue samples were collected from each patient, including: (1) one sample prior to AIT treatment, (2) a second sample after AIT and hormone replacement therapy (HRT) for seven days [i.e., 2.5 mg Letrozole (Novartis Canada, Dorval, Quebec) every other day and 1 mg/day Estrace (Warner Chilcott, Rockaway, NJ USA)]. Each sample was collected using Quick-Core Biopsy Needles (QC-16-6.0-20T, Cook Medical, USA) after local intradermal injection of 0.5 mL of 2% Xylocaine (AstraZeneca, Wilmington, DE). All samples were taken from the right breast at the 10 o’clock position, 5 cm from the border of the areola. The samples were stored in microcentrifuge tubes at -80°C until use. Prior to analysis,
each sample was transferred to a fresh microcentrifuge tube where it was weighed and then homogenized by grinding with a disposable polypropylene pestle (VWR, ON, Canada), while kept on dry ice. Samples were further stored on dry ice until analysis by on-chip immunoassay (as above). Raw and corrected amounts of E2 in each sample were determined as in rat-tissue experiment (3).

4.3. Results And Discussion

4.3.1. Integrated Method Development

The primary goal of this work was to develop a portable platform that allows quantification of estradiol (E2) in core needle biopsy (CNB) samples in ~40 minutes, a duration chosen to allow for acquisition of results during a visit to the physician, as in Figure 4.1. As described in the introduction, this goal was motivated by the need to evaluate the tissue-specific efficacy of aromatase inhibitor therapy (AIT), which is prescribed to post-menopausal patients as both a front-line treatment and as an adjuvant therapy for ER-positive breast cancer. In Chapter 2, we demonstrated that digital microfluidics (DMF) can be used to evaluate E2 and other steroids in CNB samples, unfortunately, that method relies on analysis by HPLC-MS/MS, which is not compatible with the primary objective of this work (above).

Here, we describe E2 quantification in CNB samples by means of digital microfluidic immunoassays, using an instrument the size of a large shoebox (18 x 23 x 30 cm), shown in Figure 4.1C. The system includes an integrated photomultiplier and step-motor-mounted magnet, and was originally developed for DMF immunoassays in liquid samples (e.g., serum or buffer). Here, we have extended the DMF immunoassay workflow to accommodate solid CNB samples, through a complex two-stage procedure comprising: (1) a six-step analyte extraction stage, and (2) a sixteen-step analysis stage. In initial work, the system was optimized for stage (1), which is depicted in Figure 4.2(A,B). Briefly, in each experiment, a ~5 mg tissue sample is loaded onto the device, where it is extracted into 4.8 µL droplets of methanol, which are driven to a hydrophilic anchor to dry. The sample is then reconstituted in buffer for analysis, either by on-chip ELISA or
off-chip HPLC-MS/MS. The hydrophilic anchor represents an improvement on our original DMF CNB-extraction technique, reducing the drying time by 6-fold (from 30 min to 5 min). As reported previously\(^\text{202}\) for unrelated applications, a hydrophilic anchor on a DMF device allows for reproducible concentration of analytes for subsequent recovery and analysis. In addition, by defining a sacrificial area for analyte-adsorption, the remainder of the hydrophobic device surface is protected from fouling,\(^\text{203}\) prolonging device lifetime. In initial tests, tissue samples from lactating rats (which are known to have low endogenous E2 levels) were spiked with exogenous E2, extracted by DMF, and analyzed by HPLC-MS/MS (Figure 4.2C). The average percent recovery was found to be 56 ±13% (mean ± std. dev. for \(n=10\)); consistent with our previous study of CNB extraction on DMF, which reported 65% recovery, (see Chapter 2). As shown in Fig. 4.2C, the HPLC-MS/MS measurements were found to be linear (\(R^2 = 0.984\)) and reproducible (slope = 0.615, 95% confidence interval = 0.553-0.677). Note that % recovery can be improved with longer extraction times or larger extraction volumes; in this work, it was determined that 2 x 5 min extraction into 4.8 µL droplets was an acceptable compromise between extraction time and extraction efficiency (in recognition of the goal of rapid analysis for this application). Overall, the extraction and reconstitution process (stage 1) requires ~15 min from start to finish.

After optimization of on-chip extraction of CNB samples (stage 1), we turned our attention to on-chip quantification of E2 in CNB extract (stage 2). As shown in Figure 4.3, an automated, digital microfluidic sixteen-step enzyme-linked immunosorbent assay (ELISA) procedure was developed, relying on anti-E2-modified magnetic particles and chemiluminescence detection. Briefly, reconstituted CNB extract was exposed to the immunosorbent particles (steps 1-3), which were then washed (steps 4-7), mixed with E2-enzyme conjugate (steps 8-9), washed again (steps 10-13), and mixed with reporters and analyzed (steps 14-16). This process requires ~ 25 min, bringing the full duration (for stage 1 + stage 2) to ~40 min, making it suitable for POC applications.

The immunoassay technique described here, builds from a DMF E2-assay described in previous work,\(^\text{204}\) which was implemented on a manually operated platform and was applied to standards dissolved in buffer. In developing the new method, it was found that
a number of improvements were required, addressing challenges related to (1) assay performance and (2) sample compatibility. For the former improvement (1), the complex matrix evaluated here (concentrated tissue extract) was found to be incompatible with the

Figure 4.2. Digital microfluidic (DMF) extraction of estradiol (E2) from core needle biopsy (CNB) samples. (A) Cartoon depicting E2 extraction. (1) CNB sample and extraction solvent are loaded onto the device. (2-6) E2 is extracted into droplet of extraction solvent by actively moving the droplet around the sample, before drying onto a hydrophilic anchor on the top plate. (This process is repeated twice for each tissue-sample.) (B) Picture of device and CNB sample during step (2). (C) Quantification of E2 recovered from lactating rat breast tissue samples extracted on-chip followed by analysis by LC-MS/MS (as found E2 vs. spiked E2, blue diamonds, n=10). The line of regression (solid black line; y = 0.615x-2.724) has R^2 = 0.984; the dashed black lines indicate the 95% confidence interval region for these data.
previous technique,\textsuperscript{204} which relied on the droplet-additive Pluronic L64 to enhance droplet movement and prevent biofouling. In this work, the much more potent droplet-additive, Tetronic 90R4 (recently reported\textsuperscript{205} to be useful for manipulating undiluted serum) was included at 0.1\% (v/v) in all reagent and sample solutions, which allowed for robust droplet movement for the duration of the assay. For the latter improvement (2), the sample and wash-buffer incubation duration as well as the number of incubation steps were optimized to allow for quantitation of analytes over a wide dynamic range (i.e., 0-20 ng/mL here, relative to 0-1 ng/mL reported previously\textsuperscript{204}).

As far as we are aware, the immunoassay technique described here (Fig. 4.3) is the first (in any format) to be designed for direct quantification of small-molecule-analytes from core-needle-biopsy sample. Thus, a test was devised to evaluate the performance of the on-chip immunoassay relative to that of HPLC-MS/MS. Breast tissue samples from lactating rats were spiked with exogenous E2 and then measured by HPLC-MS/MS and the on-chip immunoassay. As shown in Figure 4.4, there was a strong positive correlation between the two sets of measurements ($r = 0.996$), and the relationship was reproducible across the concentration range tested (slope = 0.455, 95\% confidence interval = 0.423-0.488). The slope <1 indicates that the immunoassay has reduced recovery \% relative to HPLC-MS/MS. This was confirmed by comparing found to spiked values obtained using the immunoassay, which had recovery of 20.2± 4.6 \% (mean ± std.dev. for $n=10$). This recovery was used to correct raw measurements in specimens collected for individual analysis, described below.

The reduced recovery for the on-chip immunoassay relative to that of HPLC-MS/MS is not surprising, as an internal standard is used for the latter technique to account for sample loss during extraction. Further, it is widely understood that LC-MS/MS\textsuperscript{206} and GC-MS/MS\textsuperscript{111,207} are “gold standard” laboratory techniques for steroid hormone analysis, with advantages (relative to immunoassays) in detection limits, specificity, and multiplexing capabilities. But the integrated immunoassay described here is useful because it is portable, and the precision and correlation of the new technique to HPLC-MS/MS gave us confidence to apply the new technique to precious CNB samples, as described below.
Figure 4.3. Digital microfluidic (DMF) quantification of E2 by on-chip immunoassay. (A) Schematic of DMF device—in this view, E2 has already been extracted (as in Fig. 4.2) from the CNB (red) into extraction solvent (blue), dried onto the hydrophilic anchor, and reconstituted in sample-buffer (yellow). The other reagents include wash buffer (orange), estradiol conjugated to horse radish peroxidase (E2-HRP) (burgundy), a suspension of anti-E2 antibody-modified paramagnetic particles (white circles), a solution of hydrogen peroxide (H$_2$O$_2$) (purple), and a solution of luminol (pink). (B) Schematic of the 16-step immunoassay process, comprising (1-7) magnetic bead and sample delivery, incubation, and bead washing, (8-13) conjugate delivery, incubation, and bead washing, and (14-16) reporter/reagent delivery, incubation, and analysis by chemiluminescence. (C) Four frames from a video depicting magnetic bead mixing during step 16.
Figure 4.4. Comparison between on-chip DMF immunoassay and off-chip HPLC-MS/MS. Spiked rat breast tissue samples were extracted on-chip and then analyzed using either the DMF immunoassay (Y-axis) or HPLC-MS/MS (X-axis) (open circles, n=10). The line of regression (solid black line; $y = 0.455x - 1.074$) has $R^2 = 0.991$; the dashed black lines indicate the 95% confidence interval region for these data. Mean DMF-immunoassay method precision was 12.9% RSD.

4.3.2. Application to Measurements of Endogenous E2

Armed with the first integrated technique that allows for hormone quantification in tissue samples by immunoassay, we turned our attention to evaluating individual specimens. As a first test, endogenous E2 levels were measured in breast tissue samples collected from lactating and non-lactating rats (Figure 4.5). The hormone amounts measured using the new technique (5.7± 1.9 and 23.8± 11.9 fmol/mg; mean ± std. dev. for n=4 lactating and n=7 non-lactating animals, respectively) are consistent with what is known from previous
and the differences between the two groups are (as expected) significant ($p=0.0454$). These results gave us the confidence to apply the new method to precious human CNB samples.

Thirty CNB samples collected from 15 patients were evaluated using the new DMF-immunoassay system – two samples from each, one prior to treatment, and a second after one week of AIT (Table 4.3). A first observation from the data is that the E2 levels vary substantially, ranging from 7.3 to 156.5 fmol/mg. This is consistent with our previous observations by HPLC-MS/MS (see Chapter 2), and underscores the importance (for this application) in evaluating paired samples from the same patient. A second observation is that the patients seem to fall into three “groups” (groups 1, 2, and 3: these labels were arbitrarily assigned on the basis of the measurements). Group 1 includes six patients whose breast tissue E2 levels decreased after therapy, group 2 includes three patients whose levels remained approximately the same, and group 3 includes six patients whose levels increased.

Many more samples should be tested to make conclusions, but the groups of patient results in Table 4.3 suggest several interesting hypotheses. For example, one hypothesis is that the variations in E2 levels measured here represent natural fluctuations with time or heterogeneous E2 distribution in breast tissue. (Unfortunately, control samples serially collected one week apart from patients not undergoing AIT were not available in this study.) Alternatively, a second hypothesis is that physicians who treat patients in group 1 should feel confident that a suitable drug and dose are prescribed, while patients in group 2 might benefit from a larger dose or more frequent administration of drug, and patients in group 3 might be candidates to move to a different aromatase inhibitor (or a SERM, or other form of therapy). In a third hypothesis, administration of AIT for more than one week (or without combination with HRT) may be required for suppression of E2 in patients in groups 2-3. Either way, additional study seems merited, given the potential benefit to patients if the hypotheses 2-3 (or something like them) are borne out. In the latter case, a method similar to the one described here, that allows for rapid analysis (compatible with implementation during a visit to a physician’s office) on a portable instrument (that might be employed at the point of care) would prove to be an invaluable tool in the march towards a personalized regime for disease assessment and treatment.
Figure 4.5. Rat breast tissue samples extracted and analyzed using the integrated DMF immunoassay technique. Endogenous levels of E2 were measured in ~5 mg samples collected from lactating (n=4) and non-lactating (n = 7) rats. Error bars represent ± 1 S.D.
Table 4.3. Estradiol values measured using the integrated DMF method with on-chip immunoassay in core needle biopsy (CNB) tissue samples collected from fifteen postmenopausal subjects before and after treatment with AIT.

<table>
<thead>
<tr>
<th>Patient group (determined arbitrarily from results)</th>
<th>E2 level in breast tissue CNB sample (fmol/mg) before AIT</th>
<th>E2 level in breast tissue CNB sample (fmol/mg) after AIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35.3</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td>17.5</td>
<td>14.8</td>
</tr>
<tr>
<td></td>
<td>69.8</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>62.1</td>
<td>27.9</td>
</tr>
<tr>
<td></td>
<td>40.9</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>43.7*10^4</td>
<td>23.9</td>
</tr>
<tr>
<td>2</td>
<td>44.5</td>
<td>47.9</td>
</tr>
<tr>
<td></td>
<td>32.7</td>
<td>32.9</td>
</tr>
<tr>
<td></td>
<td>26.9</td>
<td>30.3</td>
</tr>
<tr>
<td>3</td>
<td>73.0*10^-1</td>
<td>44.1</td>
</tr>
<tr>
<td></td>
<td>35.7</td>
<td>50.5</td>
</tr>
<tr>
<td></td>
<td>30.8</td>
<td>156.5</td>
</tr>
<tr>
<td></td>
<td>18.7</td>
<td>112.5</td>
</tr>
<tr>
<td></td>
<td>15.6</td>
<td>67.7</td>
</tr>
<tr>
<td></td>
<td>21.4</td>
<td>31.0</td>
</tr>
</tbody>
</table>

Mean DMF method precision (based on data obtained from processing rat breast tissue samples) was 12.9% RSD.

4.4. Conclusion

The new method reported here, which relies on digital microfluidics for sample extraction and magnetic particle-based immunoassay for analysis, is the first technique capable of quantification of small-molecule-biomarkers in core needle biopsy samples on a miniaturized platform. The results described here are preliminary, but if follow-up testing supports the utility of this approach, a wide range of applications is possible, including a personalized regime for managing aromatase inhibitor therapy in postmenopausal cancer patients.
Atypical maturation of the endometrium is a major cause of infertility. This condition typically arises when the tissue fails to respond to the stimulation effects of estrogen, progesterone, or both. This can compromise implantation for in vitro fertilization, and may lead to early pregnancy loss. Unfortunately, the conventional diagnostic methods used to evaluate endometrial maturation are inadequate, which motivated the development of the techniques described here. Here, we introduce the first technique allowing for quantitation of small-molecule analytes directly in mg-size endometrial tissue biopsies. A DMF-HPLC-MS/MS assay was developed for key hormones important in endometrial maturation, and then validated against a series of ~5 mg human endometrial tissue samples to test method consistency and evaluate the extraction efficiency. This chapter represents a snapshot of a work in progress; we anticipate that additional work in the near future will complete the story.

5.1. Introduction

In vitro fertilization (IVF) is the process in which sperm is used to fertilize an egg outside of the body. Embryo transfer (ET) is the technique in which eggs fertilized by IVF are implanted into the uterus of a mother. Together, IVF and ET have become enormously successful in overcoming many different causes of human infertility. But despite decades of experience optimizing IVF and ET, the rate of successful implantation remains low, a problem that has been postulated to be caused by poor endometrial maturation.\(^{210}\)

Endometrial development is a multiphase process. The initial stage is triggered by the stimulating action of estrogen in the proliferative phase of the menstrual cycle, which also acts to prime the tissue to respond to progesterone. This estrogen-driven effect persists until ovulation, at which point the endometrial lining reaches its greatest thickness and rate of proliferation. After this stage, endometrial tissue development is primarily driven by progesterone (which is generated by the corpus luteum after ovulation during the luteal phase), which helps to convert the endometrium into a
Finally, estrogen and progesterone work synergistically to support embryo growth once implantation occurs. Inadequate hormonal stimulation at any of these stages can lead to a poorly developed endometrium associated with risk of implantation failure and recurrent pregnancy loss.

The evaluation of endometrial maturation and receptivity is conventionally done by measuring the concentrations of circulating (serum) sex steroids and through histological dating of endometrial biopsies. But there is speculation that serum concentration of steroids may not be a true representation of the intra-uterine steroid concentration, leading to inaccurate prediction of endometrial environment status. Furthermore, while histological dating provides information on cellular morphological characteristics, it has been shown to be un-reliable for detecting the quality of luteal function. Thus, the standard tools used to evaluate endometrial tissue receptivity are imperfect. We propose that quantifying the local, endometrial tissue-specific hormone profile may provide an alternative tool for functional evaluation of endometrial tissue maturation and receptivity for embryo transfer.

In this chapter, we introduce the first technique allowing for quantitation of small-molecule analytes directly in mg-size endometrial tissue biopsies. The method relies on digital microfluidics (DMF) for sample extraction and clean-up, coupled with custom HPLC-MS/MS methods that were developed to quantify androstenedione (AD), testosterone (TS), progesterone (PG), hydrocortisone (HC), epinephrine (EP) and nor-epinephrine (NE) employing positive electrospray ionization (+ve ESI) mode and estradiol (E2), triiodothyronine (T3) and L-thyroxine (T4) employing negative electrospray ionization (-ve ESI) mode. This chapter describes a work in progress that is not complete; however, we propose that the preliminary data described here suggest that the method may someday be useful for functional evaluation of endometrial tissue growth and maturation prior to IVF and ET.
5.2. Experimental

5.2.1. Reagents and Materials

Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON), including 1,3,5-estratriene-3,17β-diol (estradiol, E2), (R)-4-(2-amino-1-hydroxyethyl)-1,2-benzenedi (n)-norepinephrine, NE), (R)-(−)-3,4-dihydroxy-α-(methylaminomethyl) benzyl alcohol ((−)-epinephrine, EP), 3,3′,5-triiodo-L-thyronine (T3), 3,3′,5,5″-tetraiodo-L-thyronine (L-thyroxine, T4) and 4-pregnen-11β,17α,21-triol-3,20-dione (hydrocortisone, HC). (17β)-estra-1,3,5(10)-triene-3,17-diol-d4 (deutroestradiol-d4, E2d4), 4-androsten-3,17-dione (androstenedione, AD), 4-androsten-3,17-dione-2,2,4,6,6,16,16-d7 (deuteroandrostenedione-d7, ADd7), 4-androsten-3-17β-ol-3-one (testosterone, TS), 4-androsten-3-17β-ol-3-one-16,16,17-d3 (deuterotestosterone-d3, TSd3), 4-pregnen-3,20-dione (progesterone, PG), and 4-pregnen-3,20-dione-2,2,4,6,6,17α,21,21,21-d9 (deuteroprogesterone-d9, PGd9) were purchased from Steraloids (Newport, RI). (±)-Epinephrine-d3 (N-methyl-d3) (deuteroepinephrine-d3, EPd3), (±)-norepinephrine-2,5,6,α,β,β-d6 HCl (deuteronorepinephrine-d6, NEd6) and 4-pregnen-11β,17α,21-triol-3,20-dione-9,11,12,12-d4 (deuterohydrocortisone, HC-d4) were purchased from CDN isotopes (Pointe-Claire, QC). Triiodothyronine-[13C6] hydrochloride (T3-13C6) and thyroxine-[L-Tyr-d5] hydrochloride (deutrothyroxine,T4d5) were purchased from IsoSciences (King of Prussia, PA). All solvents were HPLC grade, and all other chemicals used were of the highest grade available.

Solvent 1 was formed from a mixture of ascorbic acid (10 mg/mL) and formic acid (20 mM) dissolved in DI water with 95% methanol. Solvent 2 was formed from NH₄OH (50 mM) dissolved in methanol. Solvent 3 was formed from a mixture of ascorbic acid (10 mg/mL) and formic acid (20 mM) dissolved in DI water with 5% methanol. Solvent 4 was formed from NH₄OH (50 mM) dissolved in DI water with 50% methanol.

For analytes quantified by +ve ESI mode, a stock solution of each analyte (1 mg/mL) was formed in solvent 1. For analytes quantified by -ve ESI mode, a stock solution of each analyte (1mg/mL) was formed in solvent 2. Four working solution mixtures were
prepared from stock solutions by serial dilution as follows: solution mixture (L1) contained the six standard “light” hormones AD, TS, PG, EP, NE and HC (100 µg/mL ea.) dissolved in solvent 1. Solution mixture (H1) contained the six standard isotope-labeled “heavy” hormones ADd7, TSd3, PGd9, EPd3, NE d6 and HCd4 (100 µg/mL ea) dissolved in solvent 1. Solution mixture (L2) contained the three standard light hormones E2, T3 and T4 (100 µg/mL ea.) dissolved in solvent 2 and solution mixture (H2) contained the three isotope-labeled heavy standard hormones E2d4, T3-13C6 and T4d5 (100 µg/mL ea.) dissolved in solvent 2. All stock and working solutions were stored at −20°C until use.

5.2.2. DMF Device Fabrication, Assembly, and Operation

Devices were fabricated, and operated under procedures similar to those described in Chapter 2 (section 2.2.2), a key difference here is represented in device assembly that was done by joining a top and bottom plate with a spacer formed from 2 pieces of 3M Scotch double-sided tape (St. Paul, MN) with a total spacer thickness of 180 µm.

5.2.3. Porous Polymer Monolith Formation

Porous polymer monolith discs were prepared under procedures similar to those described in Chapter 2 (section 2.2.3).

5.2.4. Endometrial Tissue Samples

Ethics approval for this study was granted by Mount Sinai Hospital. Participants with regular ovulatory cycles undergoing natural cycle monitoring were recruited for the study. Each prospective participant was fully informed regarding details of the research, and agreed to participate by submitting a signed informed consent form. For each prospective subject, a basic pelvic ultrasound was performed on day 1, to exclude those with residual cyst retention or inadequate endometrial shedding. After enrollment, a pelvic ultrasound was done on the tenth day of the cycle to ensure dominant follicle formation. On the sixth day post-ovulation, an endometrial suction catheter was introduced into the uterine cavity without anesthesia to obtain primary endometrial tissue specimens. The specimens were then stored in sterile cryotubes at -80°C until use.
Prior to analysis by DMF-LC-MS/MS, each primary specimen was thawed and sectioned into at least two sub-samples. Each sub-sample was transferred to a microcentrifuge tube, where it was weighed and then homogenized by manual grinding with a disposable polypropylene pestle (VWR, ON, Canada). This procedure was performed on dry ice, and samples were maintained on dry ice until analysis.

Endometrial tissue samples were used for three types of experiments. In experiment (1), to evaluate analyte concentration consistency, a large primary tissue specimen was sectioned into three sub-samples (~10 mg each). Each sub-sample was then further sectioned into two secondary ~5 mg sub-samples (one each destined for +ve and -ve-mode ESI, respectively). After processing as above (weighing and homogenization), the sub-samples were extracted on-chip (section 5.2.5) and analyzed (section 5.2.6). In experiment (2), to evaluate extraction efficiency, a large primary tissue specimen was sectioned into five sub-samples (~10 mg each). Each sub-sample was then further sectioned into two secondary ~5 mg sub-samples. The secondary sub-samples were spiked with aliquots of dilutions of solution mixture L1 (for +ve ESI mode experiments) or dilutions of solution mixture L2 (for -ve ESI mode experiments) to the final concentrations given in Table 5.1. After processing as above (weighing and homogenization), the sub-samples were extracted on-chip (section 5.2.5) and analyzed (section 5.2.6). The concentration of endogenous analytes was determined by two methods: (a) standard addition, and (b) comparison to external calibration curves (5.2.7). For (a), signals were plotted as a function of spiked analyte concentration, fitted with linear regressions, and endogenous concentration determined as the intercept of the regression with the x-axis. Extraction efficiencies were recorded as the ratio of endogenous concentrations determined in (b) relative to those in (a). Finally, in experiment (3), eight primary tissue specimens (from eight different patients) were sectioned into two ~5 mg sub-samples (one each destined for +ve and -ve-mode ESI, respectively). After processing as above (weighing and homogenization), the sub-samples were extracted on-chip (section 5.2.5), analyzed (section 5.2.6), and the raw concentrations of analyte were determined by comparison to external calibration curves (5.2.7). When possible, raw values were multiplied by the reciprocal of the extraction
efficiency to determine corrected amounts; raw and corrected amounts were reported as a ratio of the absolute mass of analyte relative to sample mass.

Table 5.1. Final concentrations (ng/mL) of exogenous standards spiked into five endometrial tissue sub-samples.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Conc. 1</th>
<th>Conc.2</th>
<th>Conc.3</th>
<th>Conc.4</th>
<th>Conc.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td>0</td>
<td>5.0*10^1</td>
<td>1.0*10^3</td>
<td>2.0*10^3</td>
<td>-</td>
</tr>
<tr>
<td>EP</td>
<td>0</td>
<td>1.0*10^1</td>
<td>5.0*10^1</td>
<td>1.0*10^2</td>
<td>-</td>
</tr>
<tr>
<td>PG</td>
<td>0</td>
<td>5.0*10^3</td>
<td>1.0*10^4</td>
<td>1.5*10^4</td>
<td>2.0*10^4</td>
</tr>
<tr>
<td>HC</td>
<td>0</td>
<td>1.0*10^1</td>
<td>5.0*10^1</td>
<td>1.0*10^2</td>
<td>-</td>
</tr>
<tr>
<td>E2</td>
<td>0</td>
<td>1.0*10^3</td>
<td>2.0*10^3</td>
<td>4.0*10^3</td>
<td>-</td>
</tr>
</tbody>
</table>

5.2.5. On-Chip Tissue Extraction

For each experiment, a tissue homogenate and a PPM disc were positioned on a DMF bottom plate, 25 µL of lysing solvent (dichloromethane:acetone 80:20) was pipetted onto the sample and allowed to evaporate (~30 s), and then the device was assembled with a top plate. 12.5 µL of a dilution of mixture H1 (ADd7, TSd3, PGd9, EPd3, NEd6 and HCd4, 200 ng/mL ea. in solvent 1) for +ve ESI mode experiments, or 12.5 µL of a dilution of mixture H2 (E2d4, T3-^{13}C6 and T4d5, 200 ng/mL ea. in solvent 2) for -ve ESI mode experiments was loaded into a reservoir, and the entire volume was driven onto the tissue sample. The droplet was moved across and around the tissue sample in a clockwise circular motion for 10 min, after which the sample-extract droplet was driven away from the tissue sample and then delivered to the PPM disc for clean-up. (Prior to this step, the PPM disc was activated by dispensing a droplet of methanol, delivering it to the PPM disc, incubating for 2 min, and driving the remaining methanol to waste). The sample-extract droplet was actuated back and forth across the PPM disc for 2 min before it was driven away to a collection reservoir. The process described above was repeated two times, and extract-droplets were pooled and allowed to dry at room temperature for ~10 min. The top plate was removed, and the dried samples were reconstituted in a 50 µL
5.2.6. HPLC-MS/MS

Target analytes were measured from standards or tissue extracts (generated on-chip, as above). 20 µL samples were loaded from 250 µL polypropylene vials (Agilent Technologies, Santa Clara, CA) positioned in a 54-vial plate via a CTC Analytics Leap HTS PAL Autosampler (Alexandria, Virginia). Chromatographic separations were performed using an Agilent Technologies 1100 series HPLC system (Santa Clara, CA), HPLC-MS/MS analysis was run in two different modes: (1) +ve ESI mode for detection of AD, TS, PG, EP, NE and HC, or (2) -ve ESI mode for detection of E2, T3 and T4. The former (+ve mode) was run on a Phenomenex Kinetex Phenyl-Hexyl column (2.1 mm i.d. x 100 mm long, 1.7 µm particle dia.) protected by a SecurityGuard™ ULTRA cartridge (2.1 mm i.d. x 2 mm long, 2 µm particle dia., Phenomenex) and an in-line filter (2.1 mm dia., 0.2 µm pore dia., Agilent). The HPLC was operated at 60°C temperature in gradient elution mode. The gradient started at a flow rate of 0.1 mL/min with 95% mobile phase A (DI water) and 5% mobile phase B (acetonitrile) which was held for 5 min then linearly changed to 80% mobile phase B over 2 min, and then changed linearly again to 80% mobile phase B over the next 1 min. The flow rate was then increased to 0.15 mL/min and mobile phase B was changed linearly to 100% over the next 1 min, and then held at 100% for another 1 min. The flow rate was then decreased to 0.1 mL/min and mobile phase B was decreased linearly to 5% over 1 min. Finally, mobile phase B was held at 5% for 11 min, for a total run-time of 22 min.

The latter (-ve mode) was run on an Agilent Zorbax Eclipse Plus C18 column (2.1 mm i.d. x 100 mm long, 1.8 µm particle dia.) protected by a C18 Zorbax guard column (2.1 mm i.d. x 12.5 mm long, 5 µm particle dia., Agilent) and an in-line filter (2.1 mm dia., 0.2 µm pore dia., Agilent). The HPLC was operated at ambient temperature in gradient elution mode at a flow rate of 0.2 mL/min. The gradient started with 50% mobile phase A (DI water) and 50% mobile phase B (acetonitrile), changed linearly to 80% mobile phase B over 1 min, and then changed linearly again to 100% mobile phase B over the next 2
min. Mobile phase B was then held at 100% for 5 min before decreasing linearly back to 50% over 2 min and holding at 50% for 12 min, for a total runtime of 22 min.

HPLC eluent was interfaced into an API4000 triple stage quadrupole mass spectrometer (ABSciex, Foster City, CA, USA) via an electrospray ionization (ESI) source. The ion spray voltage, declustering potential, entrance potential, collision energy, collision cell exit potential, and the multiple reaction monitoring (MRM) transitions for each light and heavy analyte are given in Table 5.2. The signal for each analyte was calculated as the ratio of the area under the curve (AUC) in the MRM chromatogram for each product ion peak of each light standard relative to that of its corresponding heavy standard.

5.2.7. Quantification By External Standard Calibration

For quantification of target analytes in tissue extracts, standard calibration curves were generated before and after each set of tissue samples. Analytes detected by +ve ESI were quantified by using dilutions of solution mixture L1 and H1 in solvent 3. Briefly, dilutions of light standards were made into twelve different concentrations (0, 0.1, 0.5, 1, 5, 10, 25, 50, 100, 500, 1000 and 5000 ng/mL) from solution mixture L1 containing heavy standards at 100 ng/mL ea. Likewise, analytes detected by -ve ESI were quantified by using dilutions of solution mixture L2 and H2 in solvent 4. Briefly, dilutions of light standards were made into twelve different concentrations (0, 0.1, 0.5, 1, 5, 10, 25, 50, 100, 500, 1000 and 5000 ng/mL) from solution mixture L2 containing heavy standards at 100 ng/mL ea. After analysis, signals were plotted as a function of the concentration of the light standards, and were fit with a linear regression. A new external calibration curve was generated before and after each set of tissue extract and was analyzed, and the endogenous analyte concentration in the extract was calculated from the average regression lines from the two plots.
Table 5.2. HPLC-MS/MS conditions and multiple reaction ion monitoring (MRM) transitions for eighteen light and heavy target analytes. In cases in which two MRM transitions were monitored for a given analyte, the two intensities were summed.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MRM transition</th>
<th>Ion spray voltage (V)</th>
<th>Declustering Potential (V)</th>
<th>Entrance Potential (V)</th>
<th>Collision energy (eV)</th>
<th>Collision cell exit potential (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>287/97</td>
<td>5500</td>
<td>78</td>
<td>9</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>287/107</td>
<td></td>
<td>78</td>
<td>9</td>
<td>35</td>
<td>10</td>
</tr>
<tr>
<td>ADd7</td>
<td>294/100</td>
<td>5500</td>
<td>78</td>
<td>9</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>294/113</td>
<td></td>
<td>78</td>
<td>9</td>
<td>37</td>
<td>8</td>
</tr>
<tr>
<td>TS</td>
<td>289/97</td>
<td>5500</td>
<td>78</td>
<td>8</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>289/109</td>
<td></td>
<td>78</td>
<td>8</td>
<td>35</td>
<td>8</td>
</tr>
<tr>
<td>TSD3</td>
<td>292/97</td>
<td>5500</td>
<td>78</td>
<td>8</td>
<td>37</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>292/109</td>
<td></td>
<td>78</td>
<td>8</td>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>PG</td>
<td>315/97</td>
<td>5500</td>
<td>78</td>
<td>10</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>315/109</td>
<td></td>
<td>78</td>
<td>10</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>PGd9</td>
<td>324/100</td>
<td>5500</td>
<td>78</td>
<td>10</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>324/113</td>
<td></td>
<td>78</td>
<td>10</td>
<td>32</td>
<td>10</td>
</tr>
<tr>
<td>NE</td>
<td>170/152</td>
<td>5500</td>
<td>60</td>
<td>6</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>170/135</td>
<td></td>
<td>60</td>
<td>6</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>NEd6</td>
<td>176/158.1</td>
<td>5500</td>
<td>60</td>
<td>6</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>EP</td>
<td>184/166</td>
<td>5500</td>
<td>50</td>
<td>5</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>184/107</td>
<td></td>
<td>50</td>
<td>5</td>
<td>32</td>
<td>5</td>
</tr>
<tr>
<td>EPd3</td>
<td>187/169</td>
<td>5500</td>
<td>50</td>
<td>5</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>187/107</td>
<td></td>
<td>50</td>
<td>5</td>
<td>32</td>
<td>5</td>
</tr>
<tr>
<td>HC</td>
<td>363/121.3</td>
<td>5500</td>
<td>90</td>
<td>9</td>
<td>37</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>367/121.3</td>
<td></td>
<td>90</td>
<td>9</td>
<td>37</td>
<td>5</td>
</tr>
<tr>
<td>HCD4</td>
<td>367/121.3</td>
<td>5500</td>
<td>90</td>
<td>9</td>
<td>37</td>
<td>5</td>
</tr>
<tr>
<td>E2</td>
<td>271.5/145.5</td>
<td>-4500</td>
<td>-120</td>
<td>-10</td>
<td>-65</td>
<td>-10</td>
</tr>
<tr>
<td></td>
<td>271.5/183</td>
<td></td>
<td>-120</td>
<td>-10</td>
<td>-65</td>
<td>-10</td>
</tr>
<tr>
<td>E2d4</td>
<td>275.5/147</td>
<td>-4500</td>
<td>-120</td>
<td>-10</td>
<td>-65</td>
<td>-10</td>
</tr>
<tr>
<td></td>
<td>275.5/187</td>
<td></td>
<td>-120</td>
<td>-10</td>
<td>-65</td>
<td>-10</td>
</tr>
<tr>
<td>T3</td>
<td>650/126.8</td>
<td>-4500</td>
<td>-110</td>
<td>-10</td>
<td>-70</td>
<td>-10</td>
</tr>
<tr>
<td>T3-13C6</td>
<td>656/126.8</td>
<td></td>
<td>-110</td>
<td>-10</td>
<td>-70</td>
<td>-10</td>
</tr>
<tr>
<td>T4</td>
<td>776/126.7</td>
<td>-4500</td>
<td>-115</td>
<td>-7</td>
<td>-60</td>
<td>-10</td>
</tr>
<tr>
<td>T4d5</td>
<td>781/126.8</td>
<td>-4500</td>
<td>-115</td>
<td>-7</td>
<td>-60</td>
<td>-10</td>
</tr>
</tbody>
</table>
5.3. Results And Discussion

5.3.1. Analysis of Analytes Associated with Endometrial Tissue Maturation

The primary goal for this project was to develop means to quantify small-molecule analytes in small biopsies of endometrial tissue. Nine analytes, androstenedione (AD), testosterone (TS), progesterone (PG), hydrocortisone (HC), epinephrine (EP), nor-epinephrine (NE), estradiol (E2), triiodothyronine (T3) and L-thyroxine (T4) were chosen as test candidates, given their direct or indirect involvement in endometrial tissue growth, maturation and infertility. AD, TS, PG, HC, EP and NE are readily ionisable by positive (+ve) ESI and thus are compatible to HPLC-MS/MS analysis of trace amounts of analyte. On the other hand, E2, T3 and T4 exhibit better ionization efficiency in negative (-ve) ESI mode. Note that in the work described in previous chapters, the relatively low ionization efficiency of E2 led us to include a derivatization step prior to analysis; in the samples described here, however, it was found that the concentration of E2 was high enough to obviate the need for extra derivatization steps.

In developing the separation methods for this project, it was important to choose HPLC conditions that facilitate baseline-resolved separation of each analyte. All of the analytes exhibit a degree of hydrophobicity, a function of the presence of non-polar functional groups in their chemical structures, which motivated the use of reversed-phase C18 columns in initial experiments. For the -ve ESI-mode analytes (E2, T3 and T4), the C18 columns worked well; however, for the +ve ESI-mode analytes, it was observed that EP and NE did not interact with the stationary phase, such that these analytes eluted with short retention times and poor resolution. To overcome this problem, a phenyl hexyl column was adopted, which allowed baseline resolution and excellent peak shape for all of the +ve mode analytes. It is likely that the phenyl hexyl columns work well for EP and NE because these analytes can interact with the stationary phase via π–π interactions. An additional challenge for these analytes was the presence of their oxidizable catechol groups, which (when uncontrolled) resulted in asymmetric chromatographic peaks. To solve this problem, ascorbic acid was included in all solutions (stock solution, extraction
solvent and reconstitution solvent) to prevent partial or complete oxidation of these analytes.

After optimizing HPLC-MS/MS conditions for all analytes to achieve maximum ionization and separation efficiency, the methods were evaluated by running a series of dilutions of a mixture of native “light” hormones containing isotope-labeled “heavy” internal standards (ISs). The ratio of the area under curve (AUC) in the MRM chromatograms for each light standard product-ion peak relative to that of its complementary heavy I.S. was plotted as a function of standard concentration, and the results are listed in Table 5.3. Good linearity was observed for all nine analytes, with 8 out of the 9 $R^2$ values $\geq 0.995$ (ranging, overall, from 0.9641 to 0.9998). Likewise, the limits of detection (LODs) were deemed to be suitable for the samples used here: 16.0, 7.2, 27.7, 17.4, 19.3, 14.5, 2.7, 0.9 and 0.6 fmol for AD, TS, PG, NE, EP, HC, E2, T3 and T4 respectively.

Finally, we acknowledge that it is not optimal to run two separations for each analysis (one in +ve ESI mode and a second in -ve ESI mode). This was dictated by the relatively old-generation MS software used here; unfortunately, it did not allow for switching ionization polarities during the course of a single chromatographic separation. But this limitation is trivial to solve – newer generations of software allow this feature; thus, we propose that in future work, the analysis-time can be reduced by a factor of two.

**Table 5.3.** Calibration data for nine target analytes.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Slope</th>
<th>Intercept</th>
<th>$R^2$</th>
<th>LOD (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>8.1</td>
<td>-0.0002</td>
<td>0.9950</td>
<td>16.0</td>
</tr>
<tr>
<td>TS</td>
<td>14.0</td>
<td>0.0021</td>
<td>0.9972</td>
<td>7.2</td>
</tr>
<tr>
<td>PG</td>
<td>15.5</td>
<td>0.0411</td>
<td>0.9989</td>
<td>27.7</td>
</tr>
<tr>
<td>NE</td>
<td>12.1</td>
<td>0.0192</td>
<td>0.9998</td>
<td>17.4</td>
</tr>
<tr>
<td>EP</td>
<td>12.6</td>
<td>0.0033</td>
<td>0.9996</td>
<td>19.3</td>
</tr>
<tr>
<td>HC</td>
<td>12.7</td>
<td>0.0125</td>
<td>0.9989</td>
<td>14.5</td>
</tr>
<tr>
<td>E2</td>
<td>7.5</td>
<td>0.0003</td>
<td>0.9998</td>
<td>2.7</td>
</tr>
<tr>
<td>T3</td>
<td>13.9</td>
<td>0.0014</td>
<td>0.9997</td>
<td>0.9</td>
</tr>
<tr>
<td>T4</td>
<td>9.8</td>
<td>0.0086</td>
<td>0.9641</td>
<td>0.6</td>
</tr>
</tbody>
</table>
5.3.2. Analyte Consistency

Armed with a method for quantifying the 9 analytes to be evaluated here, we turned our attention to measuring their concentrations in endometrial tissue samples. A reasonable criticism for the work described in chapters 2-4 is the question of analyte consistency – that is, if analyte distribution is not consistent, how can the analyst be sure that a tiny (~mg-sized) biopsy is representative of the whole? In the work described in chapters 2-4, it was impossible for us to test this question, as we did not have access to large biopsies of human tissue (or multiple small biopsies collected from the same tissue-area).

In this project, we were able to address the question of analyte consistency, as some of the human endometrial tissue specimens that were collected by suction-catheter were quite large – up to ~50 mg. Thus, in a first experiment, we sectioned primary specimens into multiple ~5 mg sub-samples, taking care to handle the primary specimen as gently as possible, in an attempt to preserve any spatial heterogeneity. Each sub-sample was then homogenized, extracted, and analyzed separately. Five analytes (PG, NE, EP, HC and E2) were detectable (AD and TS levels were not assessed in this experiments while T3 and T4 levels were not detectable in tested samples), and were used to test tissue analyte consistency. The results are shown in Figure 5.1; coefficients of variation were calculated for each analyte, and were found to be 8.3, 12.4, 14.7, 16.1 and 16.1 for NE, EP, PG, HC and E2, respectively. These values indicate low variance in analyte distribution across different portions of tissue. This initial result is promising and we will extend them to test analyte consistency for AD, TS, T3 and T4 in the future, if further tests confirm this level of consistency, analysts can be confident that measuring analyte concentrations in small samples of endometrial tissue are representative of the whole. I propose that similar studies should be conducted with breast tissue (if larger samples can be obtained).
Figure 5.1. Consistency of analyte concentration in endometrial tissue. Three ~5 mg sub-samples were separated from a large primary endometrial tissue sample (one each for the analytes evaluated by +ve and -ve ESI-mode). Each sub-sample was separately extracted on-chip and analyzed by HPLC-MS/MS. Error bars represent ± 1 S.D. (The inset is a magnified reproduction of EP and HC signals).

5.3.3. Extraction Efficiency

Another advantage of the large specimens used in this project is the capacity to use standard additions to determine the extraction efficiency. That is, for the human breast tissue samples described in chapters 2-4, each CNB sample could only be utilized for a single experiment; sub-division of the sample for standard additions was not feasible. This led us to use rat-fat and rat-breast-tissue samples (which were available in larger specimen sizes) as proxies for human breast tissue samples, to estimate extraction efficiencies. In this project, the relatively large human endometrial tissue samples provided us with the flexibility to use the method of standard addition to determine extraction efficiencies directly from the matrix under investigation.

In these experiments, primary endometrial tissue specimens were sectioned into sub-samples and then spiked with standards; the final concentrations are given in Table 5.1. The spiked samples were processed, analyzed, and the measured signals for each analyte
were plotted relative to spiked concentrations (Figure 5.2). The endogenous analyte concentrations determined in this manner (by standard addition) were found to be $2.6 \times 10^2$, $7.6$, $2.3 \times 10^3$, $1.8 \times 10^1$ and $1.6 \times 10^3$ ng/mL for NE, EP, PG, HC and E2 (noting that AD and TS levels were not assessed in this experiment). The endogenous concentrations were also determined by comparing non-spiked sample signals to external calibration curves, yielding $7.8 \times 10^1$, $2.6$, $5.8 \times 10^2$, $5.6$ and $4.4 \times 10^2$ ng/mL for the same analytes, respectively. The extraction efficiencies were defined as the ratios of endogenous concentration determined by external calibration method relative to those determined by standard addition method, and were found to be 29.9, 33.9, 25.5, 30.3 and 27.9 for NE, EP, PG, HC and E2. Extraction efficiencies were not calculated for T3 or T4 because their levels were not detectable in the investigated samples.
Figure 5.2. Quantification of endogenous NE, EP, PG, HC and E2 in human endometrial tissue samples by standard addition. Each measurement (blue diamond) corresponds to a sub-sample (resected from the same primary sample) that was spiked with the analyte concentration indicated on the x-axis. Linear regressions (black lines) were generated, and the endogenous concentration for each analyte was calculated by extrapolating the curves to the x-axis-intercept.
5.3.4. Quantifying Human Samples

Finally, the validated DMF-HPLC-MS/MS platform described above was applied to quantify nine analytes in endometrial biopsy tissue specimens collected from eight different patients (Table 5.4). As shown, the raw results were determined by comparison to external calibration curves, and (when possible) corrected for the extraction efficiencies described in section 5.3.3.

As shown in table 5.4, of 72 measurements, all but 6 of them were above the limits of detection. The average amounts of each analyte across the nine endometrial samples were 0.4, 11.2, 4.2 *10^1, 0.3 and 0.2 pmol/mg and 8.1*10^1, 9.2*10^2, 6.2 and 6.6 fmol/mg for AD, PG, NE, EP, HC, TS, E2, T3 and T4 respectively. As far as we are aware this is the first report to quantify all nine molecules simultaneously in endometrial sample biopsies. Interestingly, these levels do not seem to agree with the reported distribution of the same molecules in serum of females during the luteal phase, which was reported to be 3.7, 1.6*10^2, 1.3, 4.7, 4.0*10^2, 4.0*10^-3, 2.0, 3.8*10^-3 and 22.7*10^-3 fmol/mg for AD, PG, NE, EP, HC, TS, E2, T3 and T4 respectively,\textsuperscript{215-220} noting that serum concentration was rescaled as analyte mass/serum mass, using an average serum density of 1.024 g/mL). An increase in tissue levels of all analytes in comparison with serum levels can be seen, with an exception to HC which seems to be more highly concentrated in serum, which further support our hypothesis of the importance of local hormone monitoring in tissue matrices.

This method, in which each sample is analyzed one time and results compared to external calibration curves, is fast, and may be appropriate for future work with smaller catheters that can only capture a few milligrams of tissue. But the correction applied on the basis of extraction efficiency assumes that the extraction efficiency is the same for all samples. A different analysis (without the above assumption) could be run in which each sample is measured by standard additions. That method may be more accurate, but would require longer experimental time, and is only suitable for cases in which large specimens are available.
This project is not yet complete – the next stage will be to expand the number of patient samples listed in Table 5.4 and to compare the results to IVF and ET success-rates for each patient. If a correlation exists between hormone concentrations and IVF/ET outcome, the techniques described here may represent a useful new tool for guiding physician and patient decision-making. If the ultimate correlation requires measuring many hormones, this procedure will likely always require mass spectrometry (and will remain a laboratory technique). But if one or a small number of hormone concentrations are (on their own) predictive of clinical outcome, it may be possible to develop a point-of-care system relying on integrated, on-chip ELISAs, as described in Chapter 4.

**Table 5.4.** Measured analyte amounts in endometrial tissue biopsy samples from human subjects normalized by sample mass. Values were corrected for extraction efficiency when possible.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>AD pmol/mg</th>
<th>TS fmol/mg</th>
<th>PG pmol/mg</th>
<th>NE pmol/mg</th>
<th>EP pmol/mg</th>
<th>HC pmol/mg</th>
<th>E2 fmol/mg</th>
<th>T3 fmol/mg</th>
<th>T4 fmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>1.3*10^2</td>
<td>6.1*10^1</td>
<td>1.3*10^2</td>
<td>0.4</td>
<td>0.3</td>
<td>7.8*10^3</td>
<td>1.4*10^1</td>
<td>1.3*10^1</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>9.7*10^1</td>
<td>3.3</td>
<td>3.8*10^1</td>
<td>0.4</td>
<td>0.3</td>
<td>5.3*10^3</td>
<td>2.3</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>7.4*10^1</td>
<td>1.2</td>
<td>4.0*10^1</td>
<td>0.4</td>
<td>0.1</td>
<td>1.1*10^2</td>
<td>4.4</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>3.4*10^1</td>
<td>1.3*10^1</td>
<td>1.2*10^1</td>
<td>0.1</td>
<td>0.2</td>
<td>1.1</td>
<td>4.5</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>2.1*10^2</td>
<td>9.3</td>
<td>2.3*10^1</td>
<td>0.4</td>
<td>0.6</td>
<td>4.6</td>
<td>2.0*10^1</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>6.2*10^1</td>
<td>0.9</td>
<td>2.7*10^1</td>
<td>0.5</td>
<td>0.3</td>
<td>1.8*10^1</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>0.3</td>
<td>1.7*10^1</td>
<td>1.4</td>
<td>3.8*10^1</td>
<td>0.3</td>
<td>0.1</td>
<td>2.0*10^1</td>
<td>0.8</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>0.3</td>
<td>1.9*10^1</td>
<td>0.1</td>
<td>2.9*10^1</td>
<td>0.1</td>
<td>0.1</td>
<td>4.7</td>
<td>1.3</td>
<td>ND</td>
</tr>
</tbody>
</table>
5.4. Conclusion

The lack of standardized sample handling and processing tools for functional assessment of endometrial tissue is a major limitation for the field of infertility. Here, I have demonstrated proof of principle that digital microfluidics (DMF) coupled to HPLC-MS/MS may be a useful new tool for this purpose. This section demonstrates initial work towards a fully integrated automated platform for tissue sample profiling.
Chapter 6
Concluding Remarks and Future Perspectives

6.1. Concluding Remarks

Digital microfluidics is a microscale fluid handling technique in which discrete droplets are controlled electrodynamically on the surface of an array of hydrophobic insulator coated electrodes. The portability, ease of automation, design flexibility, capacity to perform multiple complex sample processing steps simultaneously and ease of integration with detection systems has led to a growing popularity of DMF for use in lab-on-a-chip systems. A wide array of chemical analysis applications have been developed for DMF including chemical assays, biological sample processing, pharmaceutical analysis, proteomic workflows, and clinical tests. DMF has been extensively employed for homogeneous systems involving liquid-phase reagents and solutions; on the other hand, the use of DMF with solid samples has been limited to dried blood spots and dried urine spots. When I began my doctoral research, only one method had been previously reported for tissue sample processing on DMF devices; in this dissertation, I describe several DMF techniques that fill this gap, highlighting the compatibility of this technique with handling, process, and analysis of milligram-size tissue samples. I summarize these contributions below and propose suggestions for future work.

A Microfluidic Technique for Quantification of Steroids in Core Needle Biopsies (Chapter 2)

This chapter describes the development and use of the first DMF platform for the multiplexed extraction, clean-up and quantitation of steroids from CNB samples. The method was based on DMF for tissue sample extraction and clean-up coupled to off-line analysis of sample extractate with HPLC-MS/MS. The use of CNB samples as a means to bridge the gap between tissue sample processing and multiplexed small molecule quantification employing lab-on-a-chip platforms was deemed successful. Specifically, in comparison to the state of the art technique in the literature, the DMF method enabled a forty-fold reduction in solvent consumption and a thirty fold reduction in sample size.
Further, the extraction efficiencies of the DMF method were comparable to those of the macroscale technique with CVs ranging from 11.2 to 22.5%.

For DMF to fully replace current macroscale tissue processing methodologies, I propose that additional validation steps must be performed. This would include evaluation of analyte consistency across different locations of breast tissue, which can be done by collecting more than one CNB sample from each patient (e.g., see the attempt to address this problem in Chapter 5). Additional avenues for further research include expanding the suite of molecules analyzed towards comprehensive metabolome tissue profiling using DMF and increasing the number of samples analyzed per device (which would increase throughput).

The Effect Of Bilateral Salpingo-Oophorectomy On Circulating And Breast Tissue Hormone Levels, A Digital Microfluidics Approach (Chapter 3)

This chapter describes the development of an application of CNB sample processing on DMF and expands on the work introduced in Chapter 2. Oophorectomy has been associated with a reduction in breast cancer risk; however, no previous report describes the effect of the procedure on tissue levels of steroid hormones. In the work described in this chapter, we attempted to address this gap by utilizing the previously developed DMF-HPLC-MS/MS technique for extraction, clean-up and quantification of key steroid hormones (namely, E2, AD, TS and PG) from CNB samples collected from subjects prior to and following bilateral salpingo-oophorectomy. The findings suggest that oophorectomy is associated with a reduction in E2, AD and TS levels in the breast tissue in over 50% of patients, while the impact on PG levels in the breast was less clear. This further supports evidence for suitability of DMF as a platform for precious tissue sample analysis.

This study was not without limitations; the study population was small and was not sufficiently powered to conduct robust statistical analyses to adjust for potential factors that might influence sex hormone levels such as BMI or age. Nonetheless, the ability to obtain multiple breast tissue samples from the same individual prior to and following surgery highlights a unique strength of the technique – this has not been feasible in
previous work because of the invasiveness of routinely employed tissue collection procedures. I propose that future work could be developed towards increasing the number of analyzed samples to improve the statistical power of the study, and to facilitating the generation of a database for steroid hormone tissue concentration levels in different types of patients, a resource that is currently missing in literature.

**Towards A Personalized Approach To Aromatase Inhibitor Therapy: A Digital Microfluidic Platform For Rapid Analysis Of Estradiol In Core-Needle-Biopsies (Chapter 4)**

This chapter describes the first technique allowing for quantitation of small-molecule analyte directly in CNB samples on a miniaturized portable platform. Using digital microfluidics (DMF) for sample processing combined with a magnetic bead-based competitive immunoassay, this method was capable of running a complex procedure (i.e., a six-step tissue extraction, followed by a sixteen-step immunoassay) in an automated fashion with limited manual intervention, for extracting and quantifying E2 from CNB samples within ~ 40 min (a duration consistent with obtaining results during a patient-visit to a doctor’s office). The portability of the platform suggests the potential for operation outside of the laboratory for on-site analysis to guide physician decision-making as part of a personalized medicine approach to treatment of breast cancer.

The performance of the DMF-immunoassay platform was compared to that of DMF-HPLC-MS/MS, with the finding of strong positive correlation between the two methods ($r = 0.996$), the new method was tested for extraction efficiency across a wide range of analyte concentrations, finding a recovery of $20.2\pm4.6\%$. The low CV indicates high precision, while the recovery was relatively low compared to DMF-HPLC-MS/MS, potentially explained by the shorter extraction time and the lack of an internal standard for extraction in the new technique. The method was successfully applied to interrogate E2 levels in CNB breast tissue samples from female subjects before and after treatment with anti-hormone cancer therapy. In future work, I propose that the panel of analytes might be expanded; additionally, focus could be placed on implementing a full assay
Towards Comprehensive Endometrial Tissue Biomarker Quantification: A Digital Microfluidics Approach (Chapter 5)

This chapter represents a snapshot of progress in our on-going efforts to extend the utility of DMF to the analysis of multiple small-molecule analytes, and to increase the breadth of information obtained from small, "precious" tissue samples. Benefitting from the luxury of larger tissues samples for this application, additional method validation steps (relative to the work described in the previous chapters) were performed, enabling tests for analyte consistency, and the use of standard additions for a more accurate determination of the extraction efficiency. Problems arising from attempts to detect a large number of analytes with varying chemical properties were resolved by optimization of the analytical technique, including: (1) careful choice of stationary phase material to maximize analyte-stationary phase interaction for optimal resolution, and (2) protecting readily oxidizable analytes from degradation by using solvent formulations containing anti-oxidants.

The next stage in this work will be to expand the number of samples collected from patients scheduled for in vitro fertilization (IVF) and to compare the assay results to IVF success-rates. If a correlation between hormone concentrations and IVF outcome is found, the technique introduced here may eventually prove to be a useful diagnostic tool to guide physician decision-making during IVF.

6.2. Future Perspectives

DMF has been around for nearly 20 years as a liquid-handling technique; only recently has it begun to be evaluated as a tool for processing solid materials. The work described in this dissertation describes a series of new techniques relying on DMF for processing milligram-sized clinical tissue samples for quantitative analysis. The results presented here suggest that future work may be compatible with targeted or global metabolic tissue applications or point-of-care (POC) testing and personalized medicine. In the future, I
propose that other fields such as tissue proteomics and nucleic acid detection might benefit from variations of the techniques described here.

The portable DMF-ELISA analysis platform described in Chapter 4 for analyzing small molecules in CNB samples represents a first step towards introducing tissue processing into the world of POC diagnostics and personalized medicine applications. A logical progression in this area would be to extend the suite of molecules to be analyzed, to test the techniques capabilities in detecting different categories of analytes and to enable the processing of different types of tissue samples. Current point-of-care diagnostics rely on different forms of biological samples (mainly blood and urine); very limited tissue POC applications are reported due to the aforementioned limitation to tissue sampling and processing. However, in some settings, time is crucial, for example in detection of acute kidney injury biomarkers, detection of traumatic injury biomarkers, and in intra-operative surgery decision-making. In such cases absolute dependence on serum or blood biomarkers has not always proven effective. An attractive alternative to serum or blood biomarkers detection is tissue, since it can serve as a representation of the local environment of a healthy or diseased status. With the significant advancement in mg-size tissue processing described in this dissertation, variations to the described tissue-processing platforms –e.g. utilizing different types of tissue, or different categories of analytes based on the target application, could be utilized for a wide range of POC testing in tissue specimens.

The work described in Chapter 4 also illustrates an important consideration in lab on a chip analytical techniques in terms of system versatility. The recent development of an integrated DMF system that combines DMF sample processing tools together with an integrated (and motorized) photomultiplier tube for chemiluminescent detection, and a magnetic lens assembly which focuses magnetic field, is promising as it improves DMF application versatility by allowing for different types of tests to be implemented including tests where reagents –e.g. antibodies, and nucleic acids can be functionalized onto the surface of magnetic beads and probed by chemiluminescence detection. For example, several tissue biomarker detection applications e.g. immunohistochemistry and in-situ hybridization depend on localization of tissue proteins and nucleic acids through their
interaction with a complementary labeled antibodies/probes. Many of such techniques have been well established, however; some main drawbacks to such platforms arise from high cost of automation and limited flexibility i.e. some automated systems allow for limited number of reagents and/or protocols to be utilized and/or limited quantification capabilities. The DMF automation system utilized in this work, can be used to help alleviate some of these drawbacks since it has been successful in (1) processing tissue samples, (2) introducing different solvents/reagents (including but not limited to antibody coated magnetic beads) to the sample with high precision and (3) quantification of tissue biomarkers through the detection of chemiluminescent signal.

The work described in Chapter 2, 3 and 5 represent key achievements toward the validation of DMF for multiplex analysis of small molecules from complex sample matrices – namely human tissue samples. The platforms developed depend on DMF for tissue sample processing with off-line detection using HPLC-MS/MS. In our work we only utilized a fraction of HPLC-MS/MS capabilities to quantify some analytes of interest to our work; however with further optimization to the currently developed platforms, and utilization of multivariate statistical analysis tools, multiplexing capabilities could be greatly enhanced allowing for information on tens or hundreds of molecules to be collected from tissue specimens, towards comprehensive metabolomic DMF tissue profiling applications.\(^{30}\)

In the work described in this dissertation, freshly frozen tissue samples were utilized for biomarker quantification; however, tissue stabilized by fixation is an attractive alternative for the future. As described in Chapter 1, tissue samples stabilized by fixation have been extensively used in the clinical laboratory for a wide range of applications, with a focus on biomarker identification. Applications quantifying small molecules in fixed tissue samples are limited and still at their infancy. Given the wide availability many such tissue specimens through tissue-banks (where fixed tissue samples are stored and correlated with known patient histories and outcomes), the development of DMF protocols to process and quantify small molecules in fixed tissue would be useful. In a recent report by Huan et. al.\(^{35}\), fixed prostate tissue biopsies were successfully processed for comprehensive metabolite coverage. Some limitations to the reported technique arise
from prolonged tissue solvent incubation time (~ 2 h), and the need to perform several manual sample processing step e.g. mixing, splitting and labeling. I propose that inherent DMF capabilities can be used to overcome such problems for example DMF active mixing capabilities can be used to shorten solvent incubation time. In addition, a complex number of sample processing steps can be automated on DMF, minimizing experimental variation and increasing the precision of the analysis.

Overall, I propose that the developments presented in this thesis represent useful milestones in the development of techniques for small molecule detection in milligram-size tissue samples. With such achievement, further progress into areas of research that were not previously well covered can be made possible such as tissue metabolomics, tissue point-of-care testing and personalized medicine applications both inside and outside of the laboratory.
References

(18) Niziolek, A.; Murawa, D. Reports Of Practical Oncology And Radiotherapy : Journal Of Greatpoland Cancer Center In Poznan And Polish Society Of Radiation Oncology 2013, 18, 245-249.
(20) Rizzardi, A. E.; Johnson, A. T.; Vogel, R. I.; Pambuccian, S. E.; Henriksen, J.; Skubitz, A. P.; Metzger, G. J.; Schmechel, S. C. Diagnostic Pathology 2012, 7, 42.
(38) Myers, M. V.; Manning, H. C.; Coffey, R. J.; Liebler, D. C. Molecular & Cellular Proteomics : MCP 2012, 11, M111.015222.
(45) Han, X.; Aslanian, A.; Yates, J. R. Current Opinion In Chemical Biology 2008, 12, 483-490.
(49) Doerr, A. Nature Methods 2015, 12, 35-35.
(55) Poulsom, R. Methods In Molecular Medicine 2001, 57, 177-197.
(63) Bumgarner, R. Current Protocols In Molecular Biology / edited by Frederick M. Ausubel ... [et al.] 2013, 0 22, Unit-22.21.
(64) Brown, P. O.; Botstein, D. Nature Genetics 1999, 21, 33-37.
(76) Chaurand, P.; Latham, J. C.; Lane, K. B.; Mobley, J. A.; Polosukhin, V. V.; Wirth, P. S.; Nanney, L. B.; Caprioli, R. M. *Journal Of Proteome Research* 2008, 7, 3543-3555.
(92) Helenius, A.; Simons, K. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes* 1975, 415, 29-79.
(97) Sharefkin, J. G.; Wolfe, J. M. *Journal of Chemical Education* 1944, 21, 449.
(100) Gaikwad, N. W. *Analytical Chemistry* 2013, 85, 4951-4960.
(104) Manz, A.; Graber, N.; Widmer, H. M. *Sensors and Actuators B: Chemical* 1990, 1, 244-248.

(163) Key, T.; Appleby, P.; Barnes, I.; Reeves, G.; Endogenous, H.; Breast Cancer Collaborative, G. *Journal of the National Cancer Institute* 2002, 94, 606-616.


