

Physical Factors Regulating Human Trophoblast Invasion



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Abstract

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Pre-eclampsia, fetal growth restriction and stillbirth are major pregnancy disorders throughout the world. The underlying pathogenesis of these diseases is defective placentation characterised by inadequate invasion of extravillous trophoblast (EVT) cells into the uterine decidua. This invasion is necessary to transform the uterine arteries, ensuring an adequate maternal blood supply into the intervillous space for normal fetal growth and development. The mechanisms that regulate trophoblast invasion remains poorly understood, but it is known to be influenced by a number of factors in the uterine environment. These include interactions with maternal immune cells as well as cytokines and the products from the uterine glands.

In this thesis, physical factors, specifically, tissue stiffness and oxygen are studied as regulators of trophoblast invasion. The mechanical environment is known to regulate cell fate and the migratory behaviour of cells. Despite invasion of EVT cells through decidual tissue rich in extracellular matrix (ECM) proteins, there has been no study investigating how tissue stiffness might regulate EVT invasion. Oxygen has also long been investigated as a regulator for trophoblast invasion, but evidence is conflicting on whether low oxygen promotes or inhibits invasion. This is in part because of the wide variation in methods used and the over-reliance on trophoblast cell lines.

To examine the effects of tissue stiffness and oxygen tension, a robust *in vitro* method to examine the motility and migration of primary

EVT cells in three-dimensions (3D) was first established. This system offers significant benefits compared with two-dimensional (2D) systems used previously. Importantly, only cells expressing the HLA-G antigen, a marker for the extravillous phenotype are analysed. The stiffness of decidual tissues at the maternal-fetal interface was determined using atomic force microscopy. In patient matched samples, a 3-4 fold increase in stiffness was found where the placenta implants into the decidua, compared to where there is no implantation. Migration of single EVT cells under different matrix stiffness and oxygen concentrations in 3D were investigated. To determine whether EVT migration is directed, a microfluidic system was established, which models the oxygen gradient at the maternal-fetal interface in the first trimester of pregnancy. This system is simple and economical to set-up, and permits analysis of the migration dynamics of trophoblast cells in 3D and in real-time under different oxygen concentrations.

In conclusion, the change in stiffness at the site of implantation, is further evidence of the dramatic change that occurs in the uterine wall during pregnancy. A microfluidic system to study whether primary EVT cell invasion is directed under oxygen gradients was developed.

Declaration

I, Yassen Raad Abbas, hereby declare that this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the preface and specified in the text. This dissertation contains 58352 words and 53 figures.

Yassen Raad Abbas.

Work is my joy and my burden

-Philip Roth

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Chapter 1

Introduction

1.1 Problem statement

There are an estimated 300,000 maternal deaths each year related to complications in pregnancy and childbirth. The vast majority of these deaths occur in low income countries, with 14% the result of the hypertensive disorders pre-eclampsia and eclampsia (Say et al., 2014). In the UK, the perinatal mortality rate is 5.92 per 1,000 total births, comprising 4.16 stillbirths per 1,000 total births and 1.77 neonatal deaths per 1,000 live births (Manktelow et al., 2016). Babies that are growth restricted during pregnancy are at increased risk of still birth and those born less than 1000 g have a 20-50% risk of morbidity (Glass et al., 2015). Abnormal placental development is a leading cause of hypertensive disorders, still births and growth restricted babies. Therefore, more research is needed into the development and function of the placenta, and the mechanisms that lead to defective placentation.

1.2 Key research

A unique feature of placentation in the great apes compared to any other species is the deep invasion of placental trophoblast cells into the decidua and myometrium (Moffett and Loke, 2006). This invasion is necessary to transform the uterine arteries, ensuring an adequate maternal blood supply into the intervillous space

1.2 Key research

for normal fetal growth and development. Extravillous trophoblast cells (EVT) remodel the maternal spiral arteries resulting in highly dilated vessels, which reduces maternal blood flow resistance and increases uteroplacental perfusion to meet the requirements of the developing fetus (Pijnenborg et al., 2006). When invasion is inadequate arteries remain narrow at their opening and blood enters the intervillous space at a higher velocity, which reduces time for adequate oxygen and nutrient exchange (Burton, 2009). Inadequate invasion is associated with the “Great Obstetrical Syndromes” of pre-eclampsia, fetal growth restriction and still birth (Brosens et al., 2011).

Historically, Vaquez in 1897 was the first to observe gestational hypertension in eclamptic (onset of seizures in a woman with pre-eclampsia) women (Robillard et al., 2017). It took until the 1970’s and 1980’s for Brosens & Pijnenborg to make seminal observations of the deep trophoblast invasion that is unique to humans and that “shallow” invasion is associated with pre-eclampsia (Brosens et al., 1972; Pijnenborg et al., 1980). Over the next two decades, the term “primipaternity” was introduced exploring the relationship between occurrence of pre-eclampsia and change in paternity patterns (Robillard et al., 1993). This in part, led to investigations into immunology in placentation (Moffett and Loke, 2006). In the late 1980’s pre-eclampsia was associated with endothelial dysfunction (Roberts et al., 1989) and more recently, a distinction between early (problem of trophoblast invasion) and late onset (> 34 weeks, caused by obesity, diabetes etc...) pre-eclampsia has emerged (Roberts and Bell, 2013).

To date, numerous publications have suggested roles for a range of factors that are thought to regulate trophoblast invasion during the first trimester of pregnancy. These include: cytokines, metalloproteinases (MMPs), products of endometrial glands, uterine natural killer cells, oxygen and extracellular matrix (ECM) proteins (Xiong et al., 2013; Zhu et al., 2012; Burton et al., 2007; Hiby et al., 2004, 2010; Aplin et al., 2000). This work investigates physical factors, specifically, tissue stiffness and oxygen as regulators of trophoblast invasion.

The role of mechanics is central to basic cell functions such as motility and cytokinesis (Tee et al., 2009). In the late 1990’s, Pelham demonstrated that epithelial and fibroblastic cells change their morphology and rates of motility depending on the stiffness of the substrate they are on (Kurima et al., 1998).

1.3 Key questions and thesis outline

Seminal experiments by Engler in the late 2000's, demonstrated that naive mesenchymal stem cells (MSCs) can differentiate into bone, neurons and muscle cells when cultured on materials that match the stiffness of the native tissue (Engler et al., 2006). There are now wider questions on the role of mechanics in embryonic development and cancer (Vining and Mooney, 2017; Pathak and Kumar, 2012). In the context of trophoblast invasion, EVT cells migrate through tissue rich in ECM proteins, that is constantly being remodeled. There has been no comprehensive study looking at regulation of EVT invasion by tissue stiffness, despite our understanding of how important mechanics are to cell motility.

Oxygen has long been considered a regulator of trophoblast invasion. In the first trimester of pregnancy, the placenta develops in a low oxygen environment, which is thought to protect the fetus from reactive oxygen species (ROS) induced cellular damage (Burton and Jauniaux, 2004). In 2000, Jauniaux performed direct measurements of the oxygen concentration at the intervillous space and the decidua. A gradient of 40 mm Hg (or 5.9%) of oxygen was found but this dissipates at the onset of maternal circulation in the second trimester of pregnancy (Jauniaux et al., 2000). Numerous publications have attempted to establish whether low oxygen promotes or inhibits trophoblast invasion (Caniggia et al., 2000; Genbacev et al., 1992; Onogi et al., 2011; Fitzpatrick and Graham, 1998). However, current evidence is conflicting, in part because of the differences in methods used and the over-reliance on trophoblast cell lines, which are poor models of primary EVT (Apps et al., 2011; Abou-Kheir et al., 2017; Burleigh et al., 2007). There is a need for more studies that use primary EVT cells and an expansion of the question to look at whether EVT migration is directed in response to increasing or decreasing oxygen concentrations.

1.3 Key questions and thesis outline

This thesis aims to answer several key questions:

1. What is the tissue stiffness of human non-pregnant endometrium, pregnant endometrium (first trimester, decidua) and the placenta (first trimester)?
2. Does increasing stiffness promote EVT cell migration *in vitro*?

1.3 Key questions and thesis outline

3. Does low oxygen promote EVT cell motility?
4. Can a system using microfluidic technology be engineered to study directed migration of EVT cells under oxygen gradients that approach *in vivo* measurements?

In brief, the following chapter discusses the structure of the human placenta, the factors that regulate trophoblast invasion and current models to study trophoblast invasion *in vitro*. Chapter 3 describes the materials, methods and analysis that were used in this thesis. Chapter 4 discusses the mechanical characterisation of human endometrium, decidua and placenta using atomic force microscopy (AFM). An interpenetrating network of alginate and Matrigel to study whether increasing stiffness promotes EVT migration is described. Chapter 5 investigates whether low oxygen promotes EVT cell motility by estimating the root mean squared displacement (RMSD) of cells in culture for 24 hours. In chapter 6, the experimental design to generate and validate an oxygen gradient in a microfluidic device is explained. The final chapter (chapter 7) contains conclusions of this thesis and future directions.

Chapter 2

Literature Review

In this chapter, decidual regulation of trophoblast invasion is discussed, with emphasis on maternal cells, extracellular matrix proteins and oxygen. An overview of mechanics and tissue stiffness as a regulator of cell fate and migration is provided. The advantages and disadvantages of current *in vitro* assays to study trophoblast invasion are examined.

2.1 Biology of human placentation

2.1.1 Basic structure of the human placenta

The primary function of the placenta is to support the growth and development of the fetus. Oxygen, nutrients and waste are transported between the maternal and fetal circulations, through the placental membrane. The average diameter of the human placenta is 22 cm and the average thickness of the delivered organ at the centre is 2.5 cm (Benirschke et al., 2012). Placental tissue is primarily of fetal origin, and comprises of the chorionic plate with its villi in contact with maternal blood (Figure 2.1). Anchoring villi attach the placenta to the decidua basalis, the transformed endometrium that lines the uterus during pregnancy. The decidua is of maternal origin and is integral to successful placentation (Gude et al., 2004).

The primary cell type of the placenta is the trophoblast and it is the first cell lineage to differentiate during pregnancy. Extravillous trophoblast cells (EVT) originate at the tip of the anchoring villi and the term is used to denote trophoblast that have migrated beyond the confines of the villous trees. Maternal blood enters the intervillous space at 10-12 weeks gestation, after invasion and uterine artery remodelling by EVT cells (Moffett and Loke, 2006).

2.1 Biology of human placentation

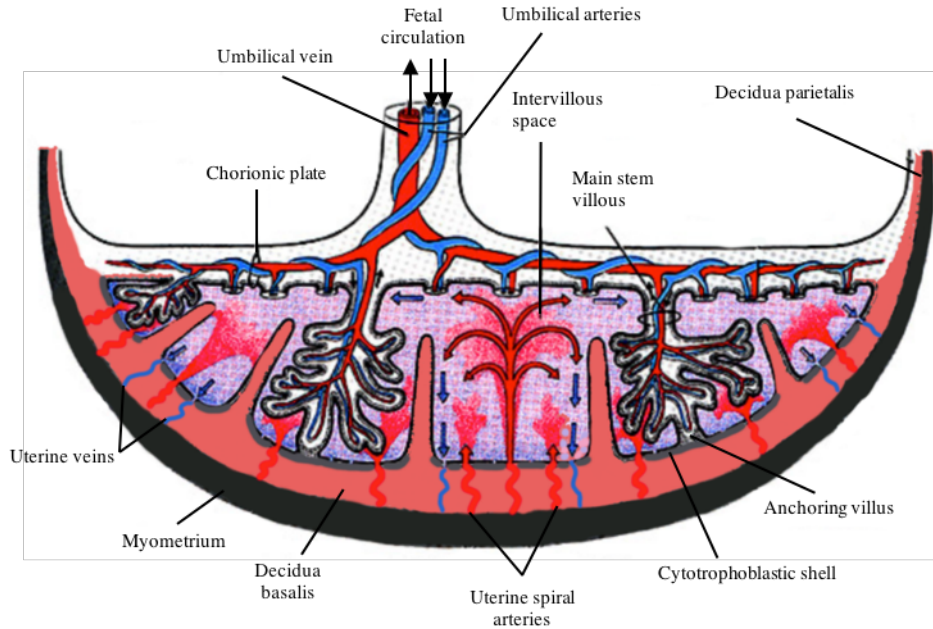


Figure 2.1: **Structure of the human placenta.** The placenta supports the growth and development of the fetus during pregnancy. Maternal circulation is established at 10-12 week gestation after adaptations of the uterine arteries by invading fetal extravillous trophoblast cells. Maternal blood enters the intervillous space and is in contact with chorionic villi, the main functional units of the placenta. The placenta provides exchange of oxygen and nutrients from the mother and removal of carbon dioxide and waste products from the fetus. Adapted from (Childbirthinternational, 2015).

2.1.2 Trophoblast invasion

Trophoblast cells develop from the trophectoderm surrounding the blastocyst and differentiate into two main lineages, villous and extravillous (Figure 2.2). The villous trophoblast form the epithelial covering of the placental villi, fusing to form syncytiotrophoblast, and EVT invade from the anchoring villi into the decidua. The physiological function of EVT is to attach the placenta to the uterine wall and remodel the spiral arteries. EVT migrate along two pathways: interstitial and endovascular (Chen et al., 2012). The process whereby interstitial trophoblast encircle spiral arteries and then destroy the arterial media that is replaced by

2.1 Biology of human placentation

‘fibrinoid’ material is unique to the great apes and humans (Pijnenborg et al., 2011). Endovascular trophoblast move down the lumen from the cytotrophoblast shell to form loose plugs and line the arteries. In the villous pathway, cytotrophoblasts differentiate into syncytiotrophoblast, which forms a continuous layer on the surface of all villous trees and is in contact with maternal blood (Knöfler and Pollheimer, 2013; Benirschke et al., 2012).

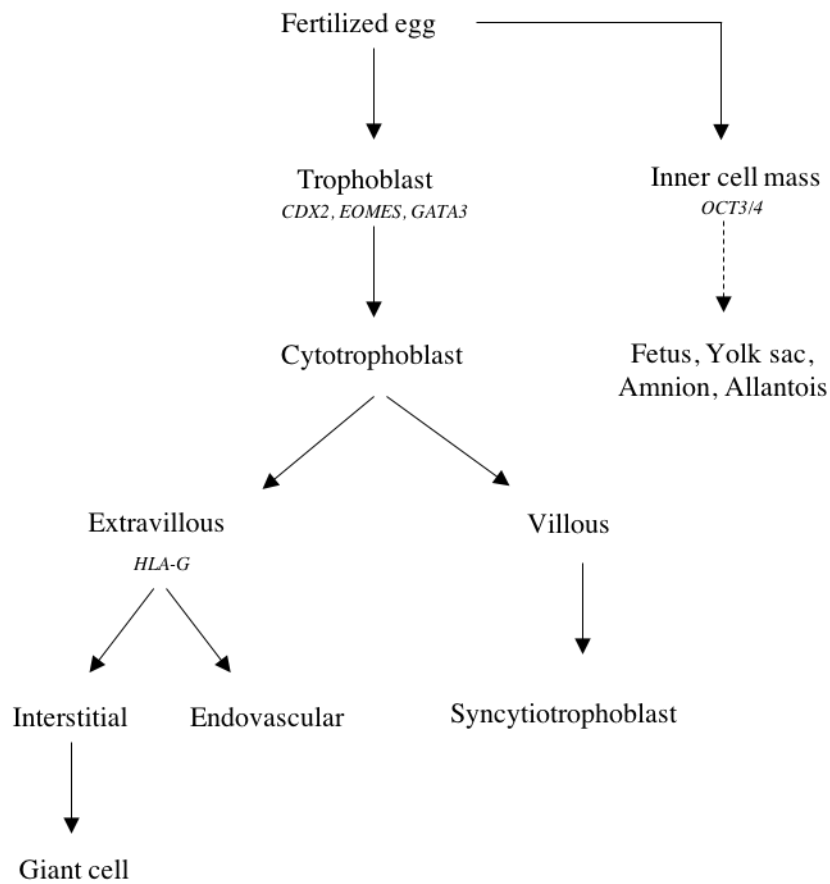


Figure 2.2: **Trophoblast cell lineage.** Trophoblasts proliferate and differentiate along two pathways - extravillous and villous. Migratory extravillous cells invade into the decidua basalis and remodel uterine arteries. HLA-G is a HLA class I molecule uniquely expressed by the extravillous phenotype. Villous cytotrophoblasts fuse to form the multinucleated syncytiotrophoblast forming the outer layer of chorionic villi in contact with maternal blood.

2.1 Biology of human placentation

Trophoblast invasion is spatially and temporally finely controlled during the first half of pregnancy. Decidua basalis contains abundant EVT by week 10 and at week 15 deep invasion of the myometrial segments is observed (Pijnenborg et al., 2006). In normal pregnancies, EVT only reach the inner third of the myometrium where they undergo transformation into multi-nucleated giant cells (Figure 2.3A) (Kemp et al., 2002). This is the final of five different phenotypes of the extravillous trophoblast (Figure 2.3B). The extent and depth of trophoblast invasion is less in certain pathological pregnancies, which results in inadequate transformation of the spiral arteries. Excessive invasion deep into the myometrium is known as the ‘placenta accreta spectrum’ and occurs when the decidua is absent or thin (Jauniaux and Jurkovic, 2012; Goldman-Wohl and Yagel, 2002). Placenta percreta, which occurs when the trophoblast extend as far as the serosal covering of the uterus, vividly demonstrates that the decidua plays a critical role in the regulation of trophoblast invasion, but the exact contribution of the various immune cells, stromal cells and other factors is still uncertain (Jauniaux et al., 2017).

2.1 Biology of human placentation

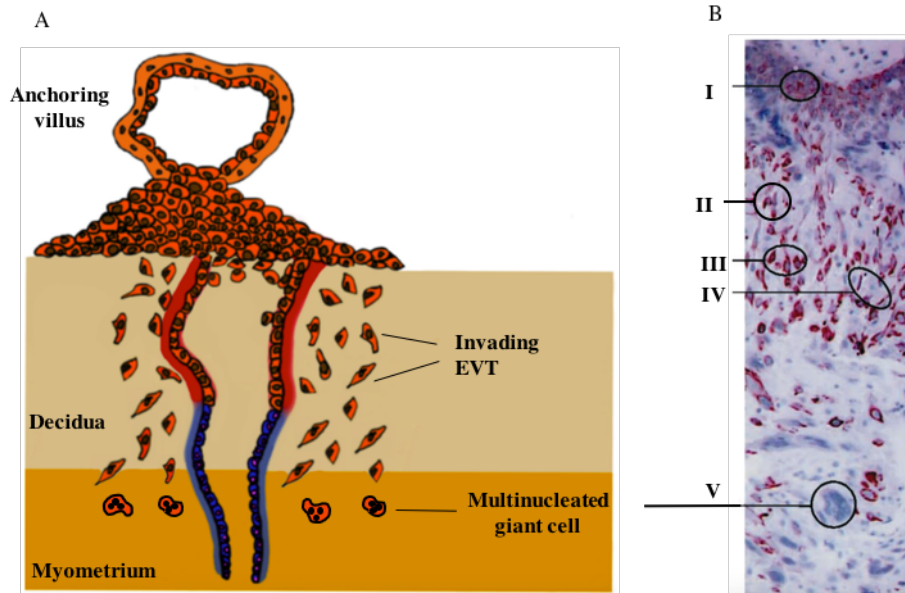


Figure 2.3: **Human trophoblast invasion.** (A) EVT cells migrate from the anchoring villus and invade into the decidua basalis. Their primary function is to remodel the uterine spiral arteries, which results in a 4-6 times increase in the diameter of the spiral artery: from high-resistance low flow vessels into large dilated vessels. (B) Histology of trophoblast invasion and the different phenotypes of the extravillous trophoblast: proliferative (not shown here), early postproliferative cells (I), large polygonal cells (II) and (III), small spindle shaped cells (IV) and multinucleated trophoblast giant cells (V). Figure 2.3B was adapted from (Kemp et al., 2002).

2.1.3 Clinical relevance of trophoblast invasion

The pathogenesis of pregnancy disorders such as pre-eclampsia and fetal growth restriction are complex but both are characterised with abnormalities in placental development. Pathological examinations of placentas from pre-eclamptic pregnancies show incomplete trophoblast invasion with extravillous cells present only in the periphery of the decidua and spiral arteries, which fail to be remodelled (Pijnenborg et al., 2006). In normal pregnancies, there is a 5-10 fold dilation at the vessel mouth resulting in a reduction in blood velocity (from 2-3 m/s to 10 cm/s) entering the intervillous space. Inadequate remodelling causes the vessels

2.2 Regulators of trophoblast invasion

to stay narrow at the opening causing blood to exit into the intervillous space in a turbulent jet like flow pattern. The exit velocity (1-2 m/s) is 10-20 times higher, causing a reduction in the residence time of blood in the intervillous space, rupturing of the anchoring villi and damage to the villous architecture (Burton et al., 2009). Impaired arterial remodelling is also thought to lead to intermittent uteroplacental circulation, resulting in hypoxia-reoxygenation of the placenta causing oxidative stress (Hung and Burton, 2006). This activates maternal endothelial dysfunction and leads to clinical manifestations such as hypertension and proteinuria from 20 weeks gestation in early onset pre-eclampsia (Hladunewich et al., 2007). Deficient remodelling also predisposes the arteries to acute atherotic changes, which will severely limit the luminal diameter and hence cause tissue hypoxia (Whitley and Cartwright, 2009; Pijnenborg et al., 2006). It is important to stress that evidence points to the quality of perfusion as the mitigating factor for abnormal placentation, not the quantity of blood flowing from maternal arteries.

Despite the strong evidence linking defective invasion and spiral artery remodelling to pregnancy disorders, there has been little progress in finding clinical solutions. The only cure for pre-eclampsia is delivery of the baby and with most pregnancy disorders, the clinical guidance is to monitor high risk patients (NICE, 2011). There is no test in early pregnancy that can accurately distinguish women who will develop pre-eclampsia despite studies into biochemical markers such as placental growth factor (PIGF), extracellular fetal hemoglobin (HbF) and soluble endoglin (sEng) (Schmidt et al., 2009; Anderson et al., 2012). The reasons for the lack of progress are complex but primarily, the mechanisms that govern trophoblast invasion are not fully understood. The field is limited by the current *in vivo* and *in vitro* models that lack physiological relevance.

2.2 Regulators of trophoblast invasion

Trophoblast invasion is a complex process involving interactions with cells of the decidua: stromal decidual cells, macrophages, decidual glandular cells and lymphocytes, notably decidual natural killer (dNK) cells (Sharma et al., 2016; Moffett-King, 2002; Burton et al., 2007; Mor and Abrahams, 2003). Numerous

2.2 Regulators of trophoblast invasion

publications have suggested roles for a range of factors including cytokines, metalloproteinases (MMPs), hormones, oxygen tension, extracellular matrix (ECM) proteins, and tissue stiffness (Zhu et al., 2012; Burton, 2009; Apps et al., 2011; Aplin et al., 2000; Abbas et al., 2017) (Figure 2.4). The diversity of these factors point to a complex network of cell signaling pathways regulating trophoblast invasion.

2.2.1 Decidual cells

Extravillous trophoblast cells are known to interact with dNK cells, which are distinctive maternal immune cells and are present in the uterine mucosal lining, the decidua, during placentation. Certain combinations of the highly polymorphic maternal KIR genes on the dNK cells and corresponding HLA-C ligands on the invading trophoblast cells result in either an inhibitory or stimulating effect on invasion. Genetic studies show that an activating dNK receptor, *KIR2DS1*, protects women from pre-eclampsia and KIR2DS1+ dNK cells promote trophoblast cell migration *in vitro* compared to women who lack this activating receptor gene (Hiby et al., 2010, 2004). Expression microarrays identified the secretion of cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) after dNK cell activation, and functional assays have shown that GM-CSF promotes trophoblast invasion *in vitro*, using primary EVT in a transwell and microfluidic assay (Apps et al., 2011; Xiong et al., 2013; Abbas et al., 2017). For other lymphocytes, evidence on their contribution to EVT invasion is limited.

Decidual glands are known to play an important role in regulating placental development in mammals. The role varies between species with significant questions remaining on their contribution to pregnancy. The glands are known to provide nutrients during the first trimester in the absence of a maternal blood supply and secrete a variety of growth factors (Burton et al., 2002). There are questions as to how these growth and other secreted factors may regulate placental morphogenesis and extravillous trophoblast invasion, but studies have been limited (Burton et al., 2007). Glycodelin, a major product of the glands, has been shown to reduce EVT invasion *in vitro* through the extracellular signal-regulated

2.2 Regulators of trophoblast invasion

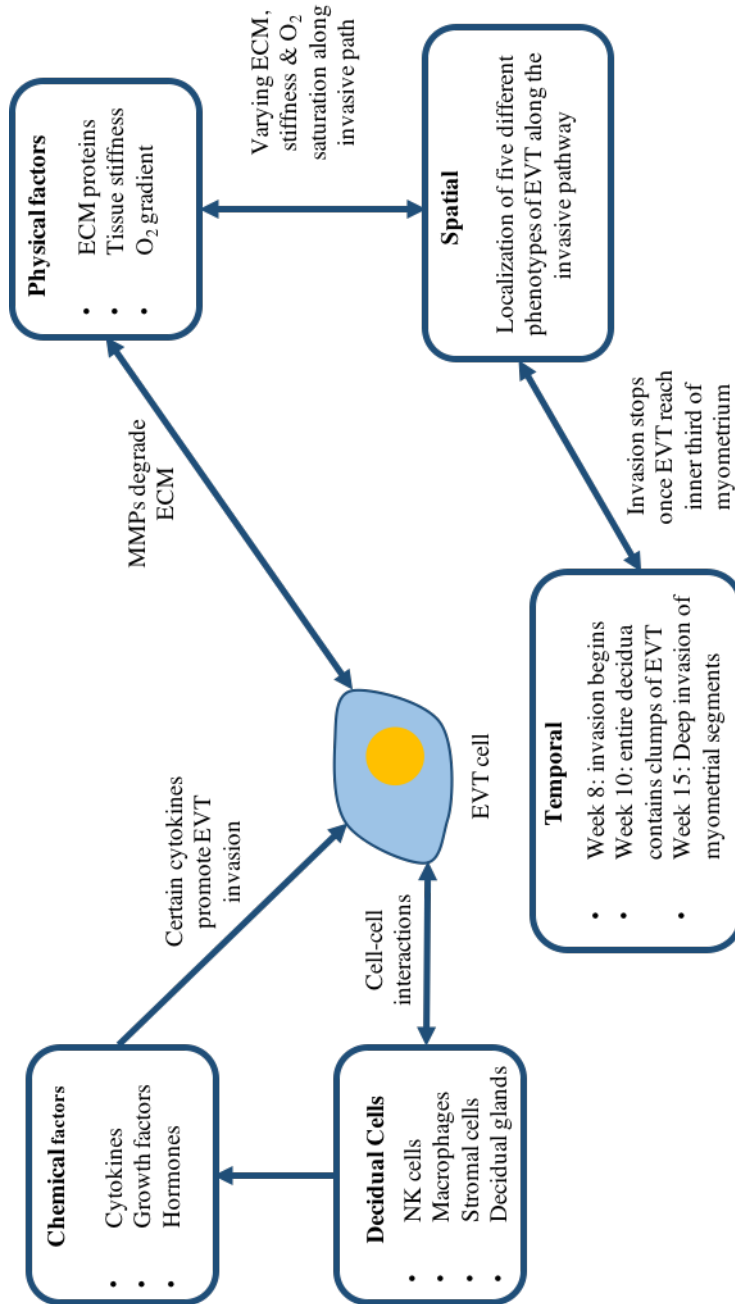


Figure 2.4: Regulators of trophoblast invasion. Extravillous trophoblasts invade the decidua coming into contact with decidual cells, where certain cell-cell interactions are known to release cytokines that promote invasion. Before the onset of maternal circulation, EVT cells are supported by secretions from the nutrient rich endometrial glands. There are physical factors that are thought to regulate invasion: the oxygen environment, ECM proteins the EVT have to cleave during migration and tissue stiffness. Trophoblast invasion is spatially and temporally controlled with varying cell populations and physical factors as EVT migrate into the decidua. Invasion begins at week 8 with deep invasion of the myometrial segments at week 15.

2.2 Regulators of trophoblast invasion

kinases (ERK) pathway (Lam et al., 2011). Despite this, it is not known if defective glandular activity is associated with pregnancy failure and complications. Model systems to study glandular activity in the human have been limited with the majority of evidence from animal models (Burton et al., 2007).

2.2.2 Extracellular matrix and tissue stiffness

The extracellular matrix (ECM) is an intricate network composed of macromolecules and is the non-cellular component present within all tissues and organs. It provides both biochemical and biomechanical stimuli that are required for tissue morphogenesis, differentiation and homeostasis (Frantz et al., 2010). Historically, research on trophoblast invasion has focussed on chemical and decidual cell regulators, with less emphasis on the role of the mechanical environment. There are two types of ECM: (1) the interstitial connective tissue matrix, which provides structural scaffolding and (2) the basement membrane, which separates epithelium from the surrounding stroma and is important in cell organisation and differentiation (Bonnans et al., 2014).

2.2.2.1 Decidual extracellular matrix

During trophoblast invasion, the decidua is rich in laminin, collagens, heparan sulfate proteoglycan and fibronectin (Kisalus et al., 1987; Aplin and Jones, 1989). These proteins are distributed pericellularly around each individual stromal cell. There is a change in ECM proteins during the menstrual cycle, which suggests their production is hormonally regulated (Figure 2.5) (Aplin and Jones, 1989; Ruiz-Alonso et al., 2012).

ECM proteins fibronectin, collagen I, III, V are distributed in the endometrium during the menstrual cycle and first trimester pregnant decidua. Collagen VI is abundant only in the proliferative phase of the cycle and progressively lost in the secretory phase and decidua. In comparison, collagen IV is up-regulated in the secretory compared to the proliferative phase, driven by progesterone production (Oefner et al., 2015). Laminin is not present throughout the proliferative phase, with a gradual increase around the periphery of each stromal cell during the secretory phase. Production of ECM is not limited to decidual cells, with strong

2.2 Regulators of trophoblast invasion

evidence that invading EVT cells secrete collagen IV resulting in higher levels at the implantation site compared to the decidua parietalis (Oefner et al., 2015).

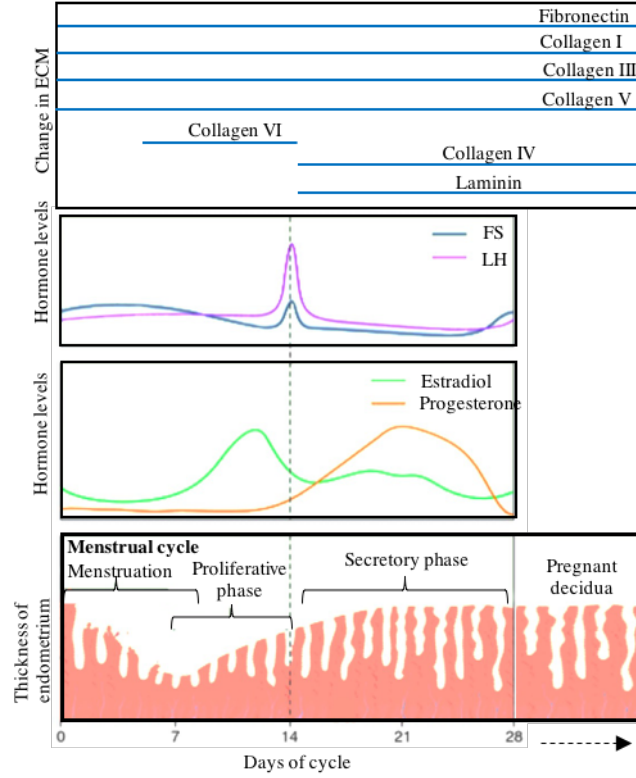


Figure 2.5: **ECM regulation by hormones during the menstrual cycle and pregnancy.** The presence of fibronectin, collagens I, III and V are constant throughout the menstrual cycle and the pregnant decidua. For collagen VI, it is progressively lost during the secretory phase. Collagen IV progressively increases during the secretory phase as progesterone levels increase. Laminin is not present throughout the proliferative phase of the menstrual cycle but gradually increases from day 14. Luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol and progesterone are hormones that regulate the menstrual cycle.

The presence of ECM in the decidua has been well characterised, but it is not clear whether ECM proteins have any functional significance other than a scaffold for the endometrium during non-pregnant life and for EVT cells to degrade and migrate through during pregnancy. The hormonal regulation of certain

2.2 Regulators of trophoblast invasion

ECM protein suggests preparation for implantation during the menstrual cycle. There is currently no evidence that pathological pregnancies or defective implantation occur due to abnormal ECM synthesis. However, this question has been understudied in trophoblast research, partly due to the difficulty in obtaining samples from pathological pregnancies during the first trimester. If there are abnormalities in ECM in the decidua, it is hypothesized that EVT invasion would be affected through a change in tissue stiffness and altered biochemical response, important in the degradation of ECM using matrix metalloproteinases (MMPs).

2.2.2.2 Tissue stiffness

Cells move through tissue using two mechanisms: (1) deformation of the cell and the space occupied by the surrounding cells and (2) by degrading ECM molecules through production of MMPs. As cells move through tissue, they probe elasticity as they anchor and pull on their surroundings using myosin-based contractility and transcellular adhesion molecules: integrins and cadherins (Discher et al., 2005). But how does a cell respond to substrates of different stiffness? *In vitro* studies show cells preferentially migrate from less to more stiff substrates, a process termed durotaxis (Isenberg et al., 2009). Cells are thought to spread more in stiffer substrates but as a consequence, the larger number of cell-matrix adhesions per cell are thought to result in a slower migration speed (Lange and Fabry, 2013). However, most studies on tissue stiffness and cell migration have been conducted in two-dimensional (2D), but there is now wide recognition that 2D substrates cannot model the complexities of the cellular environment *in vivo* (Vu et al., 2015; Wu et al., 2014).

Matrix stiffness is known to drive differentiation of mesenchymal stem cells into bone, neurons and muscle cells when cultured on materials that match the stiffness of the native tissue (Engler et al., 2006). This may have profound implications in the fields of tissue engineering and regenerative medicine. However, the mechanisms behind ECM regulation of the stem cell niche are not yet clear. In the context of placentation, there are questions as to whether ECM is important in trophoblast stem cell differentiation with particular interest in differentiation

2.2 Regulators of trophoblast invasion

along the invasive pathway. There are ECM differences where differentiation occurs with collagen IV dominant at the placental column compared to fibronectin and laminin dominant at the boundary with the decidua and the basal plate respectively (Benirschke et al., 2012). Immunohistochemistry has enabled characterisation of the ECM components but there are no data on stiffness of the first trimester placenta and decidua. The stiffness of most major human tissues has been characterised and range from the soft brain to rigid bone (Figure 2.6) (Cox and Erler, 2011; Vining and Mooney, 2017). This spectrum supports and influences the underlying cell physiology of each tissue (Irianto et al., 2016). ECM and tissue stiffness play important roles in a number of pathological or age-related conditions. For example, in cancer, a subpopulation of fibroblasts reside within the tumour microenvironment and contribute to abnormal ECM build-up and deregulate expression of ECM remodelling enzymes. There are implications in immunology and the response of immune cells to cancer with ECM dynamics having an effect on migration, activation and maturation (Lu et al., 2011).

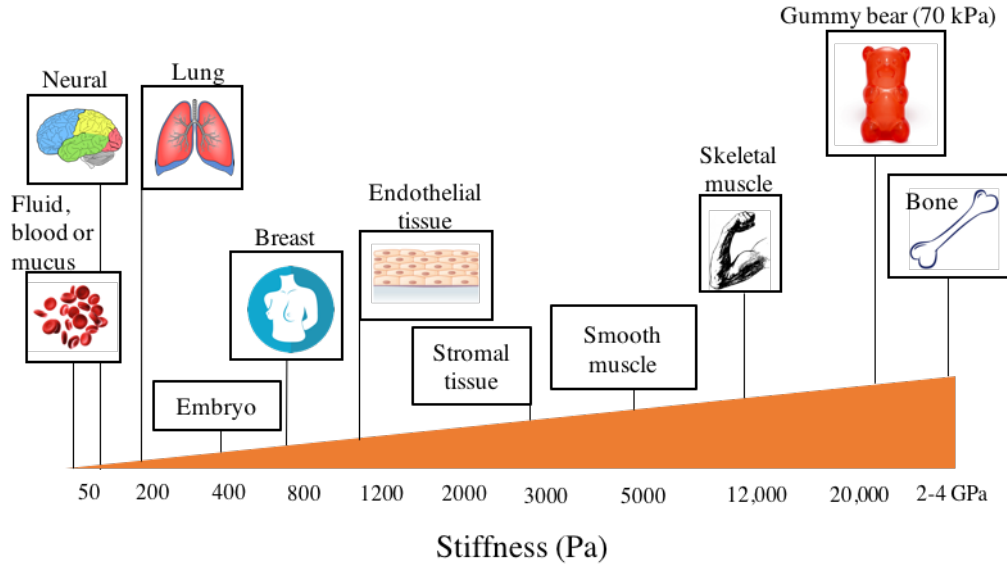


Figure 2.6: **Stiffness of human tissue.** The scale of stiffness ranges from 100 Pa for fluid, blood or mucus to 2-4 GPa for bone. Stiffness is measured as elastic modulus in pascal (Pa) and can be described as the resistance to deformation.

2.2 Regulators of trophoblast invasion

2.2.2.3 3D matrix for *in vitro* studies

In vitro migration studies of EVT are commonly carried out using the basement membrane matrix Matrigel (Corning, NY, US). Matrigel is derived from the mouse sarcoma cell line and is approximately 60% laminin, 30% collagen IV, and 8% entactin (Corning, 2012). Since stiffness is thought to regulate cell migration, it is important to determine whether Matrigel is physiological (Zaman et al., 2006). However, the continued use of Matrigel is controversial as it is mouse derived and there is batch to batch variability in protein concentration (Ross et al., 2012). The ‘black box’ nature of Matrigel may lead to experiment variability if multiple batches are used. There is a need to design synthetic gels tailored to each tissue providing information on stiffness and ECM architecture is known. Innovation in synthetic gels is being driven by the organoid field. Organoid systems enable primary cells, or stem or progenitor cells to develop into diverse, multicellular entities, which can self-renew, self-organize, and differentiate. The ‘bottom-up’ approach tailoring gels to the ECM components and stiffness of the target tissue is having profound implications in the stem cell field (Gjorevski et al., 2016).

2.2.3 Oxygen

The placenta develops in a low oxygen environment in the first trimester of pregnancy, which is thought to protect the fetus from reactive oxygen species (ROS) induced cellular damage (Burton and Jauniaux, 2004). Early human placental bed shows extensive trophoblast invasion, thus demonstrating EVT migrate under a low oxygen environment (Pijnenborg, 2000). Direct measurements from 8-10 weeks gestation of oxygen was found to be approximately 20 mm Hg (2.6 kPa or 2.5%) in the intervillous space compared to 60 mm Hg (7.9 kPa or 8.4%) at the decidua. The higher oxygen concentration in the maternal circulation is prevented from entering the intervillous space by plugs of endovascular trophoblast cells. Once the plugs dissipate the uteroplacental circulation is established at 10-12 weeks and oxygen rises to 60 mm Hg (7.9 kPa or 8.4%) at the intervillous space (Jauniaux et al., 2000).

2.2 Regulators of trophoblast invasion

2.2.3.1 Adaptations to changing oxygen concentrations

It is important to stress that even though oxygen concentrations are low in the first trimester, they are physiological and not hypoxic. There is no absolute value for hypoxia as it is a relative measure of deoxygenation (Gorr, 2017). Therefore, hypoxia and the concentrations associated with it will vary from tissue to tissue. When cells are exposed to a reduced oxygen environment they up-regulate hypoxia-inducible factors (HIF) that drive gene expression changes. The HIF pathway is an evolutionary conserved response to oxygen deprivation, a state of stress for cells. This leads to metabolic adaptations as cells change from breaking down glucose aerobically to anaerobic glycolysis as the default mode (Kim et al., 2006). But HIF also contributes to disrupting normal tissue homeostasis as the cell enters into a state of survival. At the same time hypoxic environments lead to the accumulation of ROS that cause additional stress on cellular proteins and DNA (Burton and Jauniaux, 2004).

In defective placentation, there is evidence to support the role of hypoxia in disease progression. This is a result of the disturbed oxygenation of placenta villi from inadequate remodelling of maternal arteries, as discussed in 2.1.3. However, beyond this physiology, the term is commonly misused in the placental field to describe normal low oxygen. This is true of *in vitro* studies that look at whether low oxygen drives trophoblast differentiation, proliferation and migration.

2.2.3.2 Role of oxygen in the regulation of trophoblast migration

In this study, oxygen as a regulator of trophoblast migration and invasion is investigated. The biological question is, does oxygen regulate migration of the newly differentiated extravillous phenotype from the cell column and invasion into the decidua? Cytotrophoblast proliferation at the proximal ends of the cell column occurs at low oxygen, maintained by secretions of nutrients by the endometrial glands (Burton et al., 2002). At the boundary of the cell column and decidua, where EVT migrate into the maternal tissue and invade, the oxygen concentration is higher. The current literature is conflicting as to whether low oxygen inhibits or stimulates EVT migration. The key studies are summarised in Table 2.1 and direct comparison is difficult because of the difference in the samples studied,

2.2 Regulators of trophoblast invasion

gestational age, use of ECM substrates, addition of serum, concentration of low oxygen and duration of culture. The oxygen concentrations used in culture range from 0.1% to 3%, with HIF-1 signaling at its maximum activity between 0.5-2% (Gorr et al., 2010). There are doubts as to whether cell viability can be maintained at 0.1%, despite the assurance given by the authors (Onogi et al., 2011). In addition, there are differences in whether serum is used to supplement medium. Fitzpatrick and Graham *et al.*, opting to use serum found increased motility by HTR-8/SVneo cells under low oxygen (Fitzpatrick and Graham, 1998). Serum is needed to supply plasminogen in the urokinase-type plasminogen activator (uPA) system, which is responsible for the production of enzymes that degrade extracellular matrix protein (Tang and Han, 2013). It is not clear to how important the uPA system is in EVT invasion. Culture in serum-free medium will by-pass this system and so could provide an incomplete picture of the mechanisms involved. It is important to note that there is evidence that other factors other than oxygen can regulate HIF expression. These include, hormones, cytokines and growth factors, many of which are expressed by the placenta and the decidua (Pringle et al., 2009).

2.2.3.3 Low oxygen in animal models

In normal pregnancy, questions remain on whether trophoblast cells undergo a transcriptional response to normal low oxygen and whether this is driven by up-regulation of the HIF pathway. In the human, much of the evidence is based on placental explants, however, there are questions to the effect of exposure to atmospheric oxygen immediately after tissue isolation (Caniggia et al., 2000). In contrast, *in vivo* experiments can be conducted using animal models such as the mouse and suggest a role for the HIF pathway in the pathogenesis of pre-eclampsia (Fryer and Simon, 2006; Tal et al., 2010). Other studies have focussed on the vascular changes resulting from exposure to low oxygen. One example is Rosario *et al.*, who exposed pregnant rats to 11% oxygen for between 6.5 and 13.5 days of gestation (Rosario et al., 2008). The readout was measured by immunohistochemistry (IHC) analysis of the location of trophoblast cells and the extent of arterial remodelling. The authors report increased endovascular vs. interstitial trophoblast cell invasion compared to control rats at atmospheric oxygen.

2.2 Regulators of trophoblast invasion

	(Caniggia et al., 2000)	(Genbacev et al., 1996)	(Onogi et al., 2011)	(Lash et al., 2007)	(Fitzpatrick and Graham, 1998)
Effect on migration	Inhibits	Inhibits	Inhibits	Inhibits and stimulates	Stimulates
Tissue/cell origin	Villous explants	Villous explants	Primary EVT	HTR-8/SVneo, SGHPL-4, JEG3 and JAR	HT-8/SVneo
Isolation period	5-8 wks	10-12, 16-24 wks	5-10 wks	Cell lines	Cell line
Low oxygen tension	3%	2%	Cycling between 5% and 0.1%	3%	1%
Duration	6 days	72 h	13 h	24, 48 and 72 h	24 h
Serum addition	No	No	No	Yes - 10% FBS	Yes - 5% FBS
Observation	TGF β 3 an inhibitor of trophoblast differentiation is expressed under hypoxia	Fail to invade ECM substrates due to inability to upregulate α 1/beta1	Hypoxia inhibits invasiveness through reduction of active MMP-2	Invasiveness of HTR-8/SVneo and JEG3 in 3% increased after 24 h but inhibited by 72 h. Invasion of SGHPL-4 inhibited at 3% after 24 h. JAR cells not affected by change in oxygen.	Low oxygen stimulates invasion linked to elevated expression of plasminogen activator inhibition (PAI)-1

Table 2.1: Summary of literature on the role of low oxygen on trophoblast migration.

2.3 Methods to study trophoblast motility

In addition, low oxygen was found to activate changes in uterine vasculature, with an increase in vasculature diameter observed. This study demonstrates the advantage of animal models with the ability to visualise morphological changes through IHC. However, there are questions to the effect of daily replenishment of food and water for the rats, which would cause reoxygenation of the chamber the rats are kept in. Thus, could changes be caused by hypoxia as a result of ischemia-reperfusion injury or are the changes true morphological changes?

2.2.3.4 Oxygen work

Traditionally, low oxygen work is carried out in large incubators with sophisticated gas control. Cells or explant tissues are cultured in wells with volumes ranging from 0.1-15 ml. There is controversy to whether the bulk gas concentration in the incubators, reflect the oxygen concentration cells are exposed to. Using microelectrode sensors, Chen *et al.*, found that the oxygen concentration present in the medium in a well plate is highly dependent on the depth of sampling, a limitation of static cultures (Chen *et al.*, 2013). There can also be variability in the placement of the well plate given the large bulk gas volume to be mixed, temperature variability in the incubator and location of the incubator sensor. The length of time it takes for cell medium to equilibrate to low oxygen concentrations is another limitation of this set up. Newby *et al.*, reported an equilibrium time of 4 hours when transferring 125 ml of culture medium from 18% to 6-8% and 24 h to reach 2% (Newby *et al.*, 2005). When cells are cultured long term (5-7 days) and require medium change every 2-3 days, the cells may experience a type of ischemia-reperfusion injury and the reduction to low oxygen may trigger the HIF pathway, as a response to this stress.

2.3 Methods to study trophoblast motility

2.3.1 Animal models

Placentation in mammals is enormously variable especially in the extent of invasion into the uterus by trophoblast cells. This ranges from no invasion (epitheliochorial placentation) to trophoblast cells penetrating through uterine blood

2.3 Methods to study trophoblast motility

vessels to come into direct contact with maternal blood (haemochorial placentation), with no separation by endothelium and/or epithelium (Moffett and Loke, 2006). Given this evolutionary diversity, it is important to ask whether animals can be good models for studying human trophoblast motility? The mouse is most frequently used in placental research as together with higher order primates and humans it possesses a hemochorial placenta (Schmidt et al., 2015). But there is variation in the depth of trophoblast invasion in species with a haemochorial placenta, with minimal invasion in the mouse. In contrast to human, the blood vessels of the decidua are lined by endothelium rather than trophoblasts and dNK cells are more prominent than trophoblasts in arterial remodelling (Moffett and Loke, 2006; Carter, 2007; Cross et al., 2002). The decidualized endometrium, only starts forming at implantation in mice and is restricted to the decidua basalis (Moffett and Loke, 2006).

Placentation in our closest evolutionary relatives, the great apes closely resembles human placentation in the depth and route of trophoblast invasion (Moffett and Loke, 2006). Despite limited sampling, deep invasion and arterial transformation is not restricted to humans and this has been linked to development of the brain. For the gorilla and orangutan, which are at least as large as humans, their brains are one third the size of the human brain (Herculano-Houzel and Kaas, 2011). This deep invasion, which results in the increased blood flow needed to accommodate the longer intrauterine period for development of the larger brains seems to have come at a price with the high frequency of reproductive disorders such as obstructive labour and pre-eclampsia in humans (Moffett and Loke, 2006; Hofman, 2014; Burton et al., 2015). There is little reliable evidence that primates experience these disorders, which limits their use as models for trophoblast invasion. Ethical considerations also mean that there is limited demand for primates in human pregnancy research.

2.3.2 Trophoblast cell lines

One of the limiting factors to quality trophoblast migration studies is the over-reliance on trophoblast cell lines, which is driven by the difficulty in obtaining

2.3 Methods to study trophoblast motility

primary human samples and their lack of proliferation *ex vivo*. The choriocarcinoma cell lines JEG-3 and JAR have been shown to differ substantially at the transcript level from EVT (Apps et al., 2011), while HTR-8/SVneo contain multiple cell populations, both trophoblast and stromal/mesenchymal (Abou-Kheir et al., 2017). Microarray analysis between the cell lines JEG-3 and BeWo show a markedly different expression of genes, which makes comparing experimental results between these cell lines futile (Burleigh et al., 2007). Different cell lines are known to respond differently to low oxygen, with increase in invasion (HTR-8/SVneo and JEG3), inhibition (SGHPL-4) and no effect (JAR) all reported (Lash et al., 2007). Moreover, there is little evidence that any cell line is a good model for the extravillous phenotype, with its unique invasive function and ability to form multinucleated giant cells. Although no model can ever be perfect, the repeated use of these cell lines has made it difficult to reproduce and infer what is happening physiologically. By contrast, at the same time, the cancer field, which has also posed questions on the suitability of cell lines, has reflected on failures while progressed with more successful models (Gillet et al., 2013; Burdall et al., 2003; Hughes et al., 2007).

2.3.3 Human *in vitro* assays

Immunohistological observations of the placental bed throughout gestation have provided most of the information available on placentation. Seminal observations made by Pijnenborg on pregnant hysterectomy samples have described the different invasion routes: endovascular versus interstitial (Pijnenborg et al., 1980), the contribution of interstitial trophoblast in vascular remodelling of the myometrium (Pijnenborg et al., 1983) and the extent of trophoblast invasion between normal and severe pre-eclamptic pregnancies (Meekins et al., 1994; Brosens et al., 2011). However, these provide only a snapshot and do not provide information on cellular function and mechanism. To investigate how trophoblast invasion is regulated a range of *in vitro* assays have been used that vary in their simplicity, readouts, cost and cell type (cell lines, primary cells or explant tissue) used (Table 2.2).

2.3 Methods to study trophoblast motility

Assay	Measurement	2D or 3D	Chemotaxis	HTS	Type of analysis	Advantages	Disadvantages
Scratch assay	Velocity	2D	No	Yes	Endpoint/Kinetic	Simple imaging analysis Direction of cell movement is defined Inexpensive Easy to use	Large number of cells needed Collective cell migration is observed Not suitable for chemical gradients Not suitable for non-adherent cells
Transwell (Boyden chamber)	Cell count/ labelling	3D	Yes	Yes	Endpoint	ECM components can be added Selective cytokines can be added Inexpensive Compatible with adherent and non-adherent cells	High volume to surface ratio No control of cytokine gradient Inaccurate and tedious analysis Large number of cells needed
Explant culture/Artery assay	Cell count/ labelling Migration route	3D	No	No	Endpoint	<i>In vivo</i> like microenvironment More complex than single cell or cell culture study	Maintaining long term cell viability challenging Large variability with samples Time consuming Qualitative assay Artery assays only available at term
Microfluidics	Velocity Directionality Motility	3D	Yes	Yes	Kinetic	Reduced consumption of cells Reduced consumption of reagents Flexibility of device design Real time data acquisition using live microscopy	Permeability of device material to water vapor can cause drying Experimental set-up can be time consuming Syringe pumps are expensive

Table 2.2: *In vitro* assays to study trophoblast motility. Note: HTS denotes high throughput screening.

2.3 Methods to study trophoblast motility

2.3.3.1 Wound healing assay

The wound healing assay, known also as the scratch assay, is a popular and simple 2D migration assay (Liang et al., 2007). A confluent monolayer of cells is grown on a plastic or glass substrate coated with ECM proteins and then wounded using a pipette tip (Figure 2.7A). With time lapse microscopy, closure of the wound is monitored over hours or days. Changes in cell polarity and measurement of speed and migration distance can be quantified using image acquisition software such as ImageJ (Liang et al., 2007; Jonkman et al., 2014). This assay is particularly suitable to study the effects of cell-matrix interactions and chemical factors on cell migration. The limitation of wound healing assays, however, is that they are 2D and thus of limited physiological relevance to trophoblast invasion in the decidua (Friedl and Bröcker, 2000; Cukierman et al., 2001). Evaluating single cell migration and the number of migrating cells is challenging as cells mostly migrate without losing cell-cell contacts to neighbouring cells. Moreover, there are questions over the relevance of an assay that only detects rate that cells move to fill a gap, which is substantially different to EVT invasion in 3D.

A major stumbling block in the wound healing assay is the large cell number required and this limits the use of primary HLA-G+ trophoblast cells. Trophoblast cell lines are an alternative but there are questions as to whether wound closure is caused by cell proliferation rather than migration. For example, when IL-12 was added to a monolayer of JEG-3 cells to assess its influence on trophoblast migration, cell motility was reduced as IL-12 concentration increased but this coincided with a reduction in cell number within the wound (Karmakar et al., 2004). It is unclear if IL-12 caused a reduction in motility or inhibition in cell proliferation. This experiment demonstrates the weakness of the wound healing assay as well as the problem of using proliferative cell lines.

2.3 Methods to study trophoblast motility

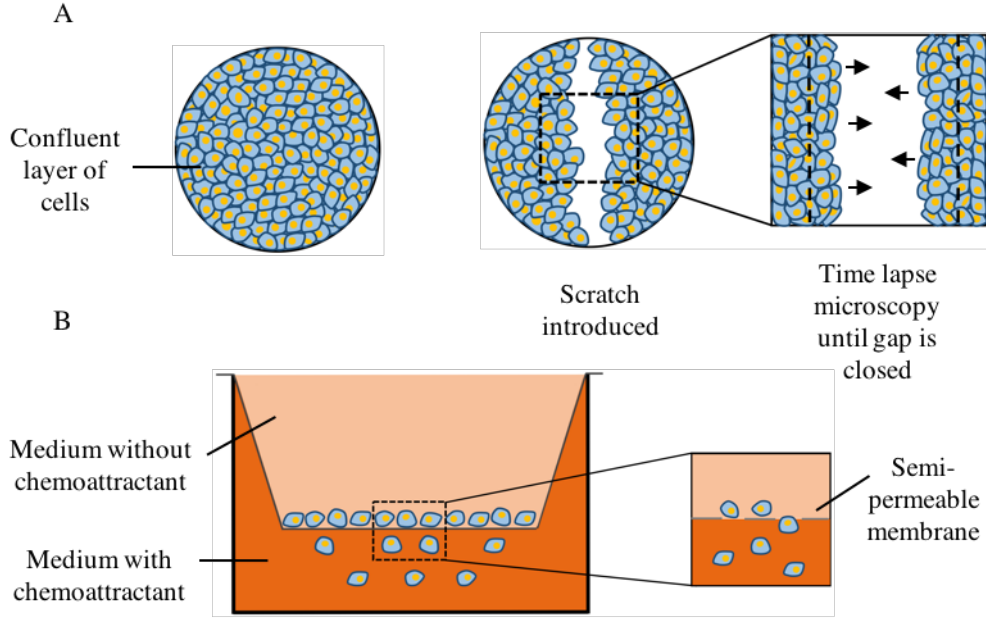


Figure 2.7: **Conventional *in vitro* assays used to study trophoblast invasion.** (A) The wound healing or scratch assay is simple, economical and 2D. A scratch is made and the cells migrate to close the gap. The rate of migration is quantified using time-lapse microscopy. (B) The Transwell or Boyden chamber is 3D and cells are seeded in an insert and placed in a well with a cytokine. Cells migrate through a semi-permeable membrane with migration quantified by counting the number of cells that have crossed the membrane.

2.3.3.2 Transwell assay

The transwell assay (Boyden chamber) has been widely used to study cell invasion. An insert containing a cell permeable membrane is placed in a multi-well tissue culture plate, with cells seeded on one side and chemoattractant added to medium on the other (Figure 2.7B). To create an invasion assay, the cell permeable membrane can be coated with a layer of ECM protein to mimic cell invasion into basement membrane or stroma. Cells are quantified with the addition of a cell-permeant dye, which is then converted into a fluorescent signal (Justus et al., 2014; Albini and Benelli, 2007). For studies with primary trophoblasts, it is necessary to reduce contamination by other fetal cells to not falsify cell migration

2.3 Methods to study trophoblast motility

results. Cells that remain on the membrane are removed using a cotton swab and migrated trophoblast cells on the lower surface of the filter are stained for a specific cell marker and counted. Using this approach Hanna *et al.*, demonstrated the promoting effect of interleukin (IL)-8 on the migration of HLA-G+ EVT (Hanna *et al.*, 2006). Jovanović *et al.*, further revealed that this is due to up-regulation of MMP-2 and MMP-9 and integrin expression (Jovanović *et al.*, 2010). Using the transwell assay, many other cytokines and hormones such as chemokine (C-X-C motif) ligand (CXCL) 16, IL-1 β , CXCL6, human placental growth hormone (hPGH) and GM-CSF were revealed to affect the migration of primary trophoblast cells isolated from first trimester placenta (Huang *et al.*, 2006; Prutsch *et al.*, 2012; Lacroix *et al.*, 2005; Xiong *et al.*, 2013).

One of the major disadvantages of the transwell assay is the simplification of the migration events with chemotaxis. Whereas the number of cells that have crossed the cell permeable membrane can be quantified, it is not possible to obtain cell speed and direction. Therefore, it is difficult to distinguish between cells crossing the membrane at high speed due to random migration and directed migration towards the chemoattractant. The stochastic nature of cell migration means comparing the effect of multiple cytokines on a cell population is challenging. To conclude one cytokine has a greater effect on cell migration than another requires kinetic readouts not possible with the transwell assay.

2.3.3.3 Explant cultures

Explant culture is a technique in which organs or small pieces of tissue are removed and cultured *in vitro*. In trophoblast research, primary villous tissue of first trimester pregnancies containing cytotrophoblast cell columns are dissected and cultured on ECM (Figure 2.8A) (Genbacev *et al.*, 1992; Bauer *et al.*, 2004; James *et al.*, 2006). Trophoblast proliferation and invasion can be studied by placing the explant tissue in the Transwell assay, where the cells remain in their appropriate cellular as well as extracellular environment. Factors that are thought to influence trophoblast invasion can be tested individually or to assess the impact of decidua secreted factors, conditioned medium from isolated decidual cells can be added (Wright *et al.*, 2006). To quantify cytotrophoblast proliferation, the

2.3 Methods to study trophoblast motility

area of explant outgrowth from the anchoring villi is determined and cells stained for markers of proliferation. The number of cells that cross the cell permeable membrane is counted to assess the effect of chemoattractant on trophoblast invasion and immunohistochemistry is used to determine if the invading cells are extravillous by staining for HLA-G (Librach et al., 1991; Wright et al., 2006).

To study maternal-fetal cell interactions and spiral artery remodelling *in vitro*, villous explants can be cultured with decidua explants on ECM proteins (Viévac et al., 1995; Dunk et al., 2003; Moser et al., 2010). The main advantage of this system is that the spatial relationship between migrating trophoblast and stroma is preserved. Explant cultures, however, have several major disadvantages. There is extensive cell death of syncytiotrophoblast, cytotrophoblast and the villous mesenchyme after 4 days of culture that could affect invading EVT (James et al., 2005). In addition, the assay can be time consuming with cultures of up to 12 days (Hu et al., 2008). Tissue of young gestational age is needed as the outgrowth decreases with increasing gestational age (James et al., 2006). Studying trophoblast migration in response to cytokines is limited given the challenges of creating a chemotactic gradient with explant tissue and without the ability to track cell movement live.

2.3 Methods to study trophoblast motility

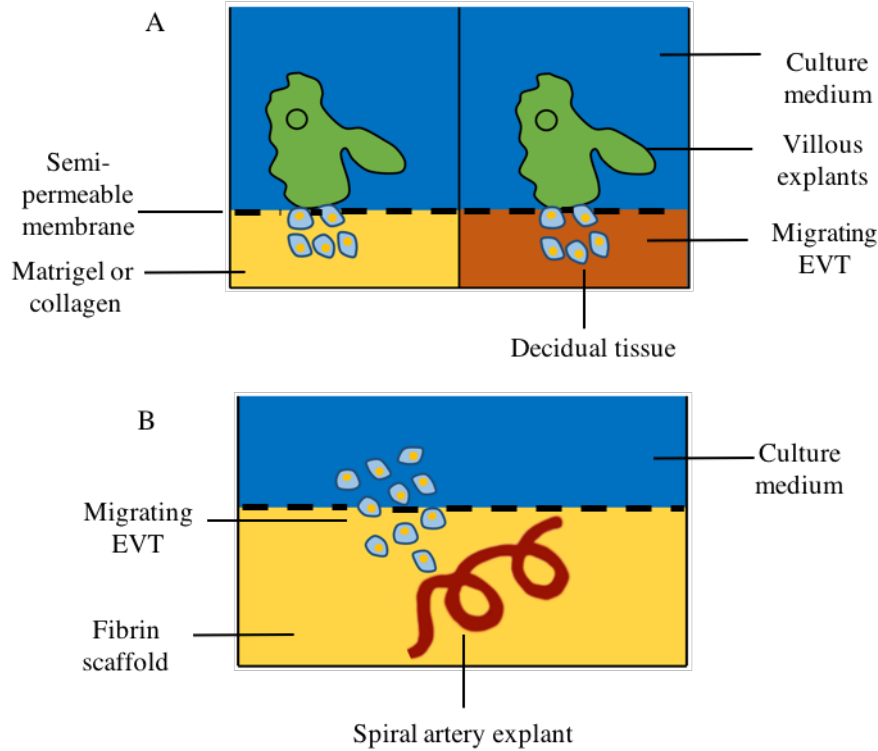


Figure 2.8: **Explant cultures and artery assay.** (A) Villous explants from first trimester pregnancies are cultured on ECM or decidual tissue and EVT outgrowth is quantified. (B) Uterine spiral arteries isolated from term pregnancies are embedded in a fibrin gel and cultured with EVT to study invasion under various regulating factors.

2.3.3.4 Spiral artery assay

Vascular remodelling of the uterine spiral arteries is critical in establishing the maternal circulation to the placenta, with impaired remodelling associated with pre-eclampsia. To observe direct interactions between EVT and cells of the spiral arteries, non-invaded arteries isolated from the myometrium of term caesarean sections can be cultured with trophoblasts (Figure 2.8B). To study interstitial invasion, the arteries are embedded in fibrin hydrogel and fluorescently labelled trophoblast cells are seeded on top and left for up to 5 days before sectioning the construct (Cartwright et al., 2002). For endovascular invasion, the arteries are mounted on a pressure myograph and perfused with trophoblast cells. Using

2.3 Methods to study trophoblast motility

this set-up Ashton *et al.*, showed that trophoblast cells lead to apoptosis and loss of the endothelial cell layer within 96 hours of co-culture (Ashton *et al.*, 2004). Crocker *et al.* suggested a role for oxygen saturation and inflammatory molecules such as $\text{TNF}\alpha$ in mediating artery transformation (Crocker *et al.*, 2005).

This assay is an attempt to study the mechanisms that control trophoblast arterial remodelling *ex vivo*, given the limitations of animal models. However, there are questions over the viability of the arterial cells and extent of ECM degradation surrounding the arteries with the long culture times. The source of the biopsies means the arteries recovered may not be a good model, given they are myometrium of non-placental bed areas at term. Given these drawbacks, it is important to ask whether the information gained goes beyond that of immuno-histochemistry on sections of the decidua and myometrium from first trimester pregnancies? Since chemical gradients cannot be generated and quantification of migration dynamics is simplified, the artery assay has limited use.

2.3.4 Microfluidic invasion assay

Microfluidics is a technology characterised by the manipulation of fluids in micrometer sized structures (Sackmann *et al.*, 2014). It has become increasingly popular in biological research because it's at the length and time scale of many biological systems. Chemical and physical factors such as cytokine and pressure gradients can be controlled in a 3D microscale environment (Figure 2.9). Rapid and low cost microfabrication techniques have led to implementation in a wide range of fields, such as organ-on-a-chip (Huh *et al.*, 2010), PCR-on-a-chip (Jha *et al.*, 2012) and single cell sequencing using droplet microfluidics (Zilionis *et al.*, 2016). Migration and invasion studies using microfluidics have been driven by the cancer field. Tumour cell invasion and metastasis lead to the formation of secondary tumours, which occurs when cancer cells penetrate the surrounding basement membrane and into the lymphatic or vascular systems (van Zijl *et al.*, 2011; Wong and Hynes, 2006). Microfluidics allows tracking of cancer cells as they invade in 3D, through basement membrane in response to chemical or physical cues. Cells can be tracked in real-time and dynamic information such as velocity and the direction of cell movement can be gained. This is particularly beneficial

2.3 Methods to study trophoblast motility

for chemotaxis studies where it is not always clear if cell migration is random or directed.

2.3.4.1 Microfluidics to study trophoblast invasion

Whilst primary human samples are preferred, the scope of migration experiments are limited by the low number of EVT cells that can be isolated from first trimester samples, typically 50-100K. With microfluidics, the small channel volumes mean only a few thousand cells are needed, therefore maximising the potential use of primary cells. Cytokines and growth factors that are thought to promote trophoblast invasion can be tested in a high throughput manner using commercial chips with multiple devices on one microscope slide. A confocal microscope can be used to image in 3D, real-time and programmed to move between chips to enable parallel experiments. Microfluidics has been adopted to study trophoblast invasion in response to GM-CSF, a cytokine produced by activated dNK cells (Abbas et al., 2017) (Figure 2.9). Trophoblast are known to interact with dNK cells, which make up 70% of the maternal leukocytes present in the decidua (Moffett-King, 2002). Certain combinations of the highly polymorphic maternal KIR genes on the dNK cells and corresponding HLA-C ligands on the invading trophoblasts result in either an inhibitory or stimulating effect on invasion (Hiby et al., 2004, 2010). Mothers with the activating *KIR2DS1* gene and an HLA-C2 fetus are less likely to have pre-eclampsia as this combination increases secretion of cytokines that promote trophoblast invasion (Xiong et al., 2013; Abbas et al., 2017). Primary EVT were found to move with increased directionality towards increasing concentrations of both human recombinant (hr) GM-CSF and GM-CSF produced by dNK cells with the activating receptor, KIR2DS1 (Abbas et al., 2017).

2.3 Methods to study trophoblast motility

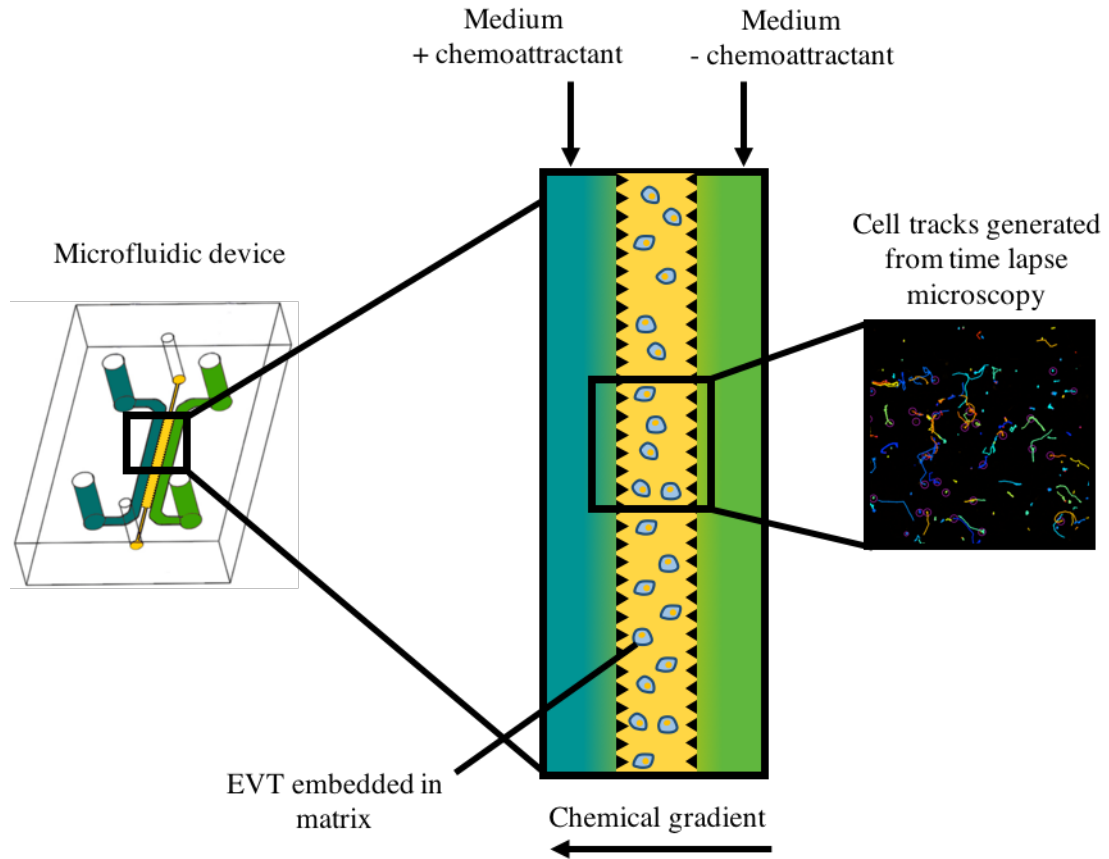


Figure 2.9: **Microfluidics assay to study EVT invasion.** EVTs are isolated from first trimester placentas, stained with a cell tracker and embedded in Matrigel in the central hydrogel channel. (a) A constant flow of medium is applied in the two side channels, either with or without a cytokine to create a gradient across the hydrogel channel. Individual cell tracks are generated from time lapse microscopy ([Abbas et al., 2017](#)).

Chapter 3

Materials and methods

3.1 Tissue samples

Samples of first trimester placental and decidual tissue were taken from routine terminations of pregnancy (6-12 weeks gestation) as previously described (King et al., 1989). Ethical approval for placenta and decidua was obtained from the Cambridgeshire 2 Research Committee (reference no. 04/Q0108/23). Secretory phase (6-11 days after pre-ovulatory luteinizing hormone surge) endometrial samples were obtained from subjects recruited from the Implantation Clinic at University Hospitals Coventry and Warwickshire National Health Service Trust with ethical approval from NHS National Research Ethics—Hammersmith and Queen Charlotte’s & Chelsea Research Ethics Committee (1997/5065). Endometrial biopsies were obtained using a Wallach Endocell sampler, starting from the uterine fundus and moving downward to the internal cervical os.

3.2 Isolation of primary cells

Handling of human tissue samples was carried out in a biosafety level 2 facility and under a biological safety cabinet. The samples were emptied into a sieve covered with a muslin gauze and washed with Ham’s F12 (biosera, Nuaille, France) medium to remove as much blood as possible. Since primary trophoblast cells absorb phenol red present in cell culture medium, Ham’s F12 medium without

3.2 Isolation of primary cells

phenol red was used during isolation and preparation of these cells. Placental and decidual tissue samples were then separated using forceps and washed further with medium. Placental villi were scraped from the membrane using a scalpel and dissociated with 0.2% trypsin (1.67 mM glucose, 0.205 M sodium chloride, 4.02 mM potassium chloride, 0.012 M disodium hydrogen orthophosphate, 2.2 mM potassium dihydrogen orthophosphate, 2 g/l trypsin and 0.2 g/l ethylenediaminetetraacetic acid (EDTA)) (Sigma, St. Louis, Missouri, US) for 9-10 min at 37 °C under constant stirring, followed by neutralisation with 10% neonatal calf serum (NCS) (Sigma, St. Louis, Missouri, US). The cell suspension was sieved through a muslin gauze and centrifuged at 500 g for 5 minutes (min) at room temperature (RT) before removal of maternal blood using lymphoprep density gradient solution (GE Healthcare, Little Chalfont, Illinois, US). Briefly, the cell solution was transferred into a universal tube with 8 ml of lymphoprep and centrifuged at 700 g for 20 min at RT, with the white band of cells collected.

3.2.1 Isolation of extravillous trophoblast cells

The cell suspension is made up of a heterogeneous population of cells that include fibroblasts, syncytiotrophoblast, mesenchymal cells, leukocytes and extravillous trophoblast (EVT). Trophoblast cells express fibronectin (FN) receptors and plating trophoblast cells at a high density leads to the adhesion of trophoblast out-competing other cells for space. Cell culture dishes (ThermoFischer, Scientific, Waltham, MA, USA) of 35 mm diameter were coated with fibronectin (FN, Becton Dickinson, Franklin Lakes, New Jersey, US). For this, 20 ml of 1 mg/ml FN in MilliQ water was diluted in 1 ml Ham's F12 by carefully and slowly pipetting up and down. Subsequently, FN was added to the dish and left at RT for 45 min to allow adhesion. The cell solution was washed once more after lymphoprep and resuspended in Ham's F12 supplemented with 20% fetal calf serum (FCS) (Sigma, St. Louis, Missouri, US), 2 mmol/L L-glutamine, 10 units/ml penicillin, 100 mg/ml streptomycin and 2 mg/ml gentamycin. This was the formulation for EVT medium (Table 3.1).

For each placental sample, one 35 mm dish was used and the dish was incubated overnight at 37 °C. Following incubation, the non-adherent and dead

3.3 Cell lines and cell culture media

cells were removed and the dish was washed 5 times with phosphate buffer saline (PBS) leaving a monolayer of EVT. For cell tracking experiments, cells were labelled by incubation at a 1/1000 dilution in cell medium with 40 μ M cell tracker orange CMTMR (ThermoFisher Scientific, Waltham, MA, USA) for 15 min at 37 °C. The monolayer was then trypsinised for 2-3 min at 37 °C until cells detached and 10% FCS was added to inactivate trypsin. The cell suspension was centrifuged at 500 g for 5 min and resuspended in 2 ml EVT medium, before mechanical agitation 60 times with a 1 ml pipette and 40 μ m nylon mesh (BD Biosciences, Franklin Lakes, New Jersey, US) to produce a single cell population. The purity of the extravillous phenotype, expressing HLA-G ranges from 40-90%, determined by Immunofluorescence and flow cytometry.

3.3 Cell lines and cell culture media

Human choriocarcinoma trophoblastic cell line JEG-3 (HTB-36) was acquired from the American Type Cell Culture (ATCC, Manassas, Virginia, US). JEG-3 cell lines were used from frozen (in liquid nitrogen) at passage 2-4. RPMI (Thermo Fisher Scientific, Waltham, MA, USA) medium was supplemented with 10 units/ml penicillin, 100 mg/ml streptomycin and 2 mg/ml gentamycin. Cell lines were kept in a humidified incubator with 5% CO₂, at 37 °C. For freezing cell stocks down, cells were trypsinised, washed and cell pellets were frozen down in a mixture of two parts dimethyl sulfoxide (DMSO, Sigma, St. Louis, Missouri, US) and three parts RPMI medium. Approx. 1×10^6 cells in a volume of 800 μ l were slowly mixed with 200 μ l of pre-cooled freeze mixture for a total volume of 1 ml per cryo vial. These were transferred immediately and stored at -80 °C overnight followed by long term storage to liquid nitrogen.

3.4 Visual detection of proteins

Cell type	Growth medium	Serum	Antibiotics	Other
EVT	HAMS-F12	20% FCS	10 units/ml penicillin, 100 mg/ml streptomycin and 2 mg/ml gentamycin	2 mmol/L L-glutamine
JEG-3	RPMI (PAA)	10% FCS	10 units/ml penicillin, 100 mg/ml streptomycin and 2 mg/ml gentamycin	2 mmol/L L-glutamine

Table 3.1: Cell culture media composition.

3.4 Visual detection of proteins

Immunohistochemistry (IHC), Immunocytochemistry (ICC) and Immunofluorescence (IF) all utilise antibodies to provide visual details on protein abundance, distribution and localisation. The technique used depends on the sample type with IHC used on tissue sections, either paraffin wax embedded or frozen, whereas ICC refers to staining of isolated or cultured cells. In IF detection, the fluorochrome is conjugated either directly or indirectly, to the primary antibody and, once excited, will emit light at a specific wavelength. In this thesis, protein detection was carried out on fixed tissue and cells, together with detection during live imaging.

The principle of IHC tissue fixation is to preserve tissue from decay while retaining antigenicity and preserve tissue architecture and cell morphology. There is a delicate balance, as over fixation destroys antigens and can result in false negative staining. After fixation the sample is incubated with blocking agents to prevent non-specific binding of antibodies and subsequently with primary and secondary antibodies. Positive staining is visualized using a chromogenic or flu-

3.4 Visual detection of proteins

orescent molecular label. For live cell imaging there is no fixation and blocking step, the fluorophore is directly conjugated to the antibody, which is incubated with the isolated cells.

With all tissue and cell staining, optimisation is needed to determine length of fixation, antibody incubation time and titration of the antibody in order to increase signal to noise ratio. The quality of the antibody will vary between manufacturers, which requires trial and error.

3.4.1 IHC and ICC - Paraffin embedded tissue samples

Decidual and endometrial samples were incubated in 4% paraformaldehyde (PFA, Sigma, St. Louis, Missouri, US) for 12 hours (h) and stored in 70% ethanol. Tissue blocks were prepared and cut into thin sections of 8 μm by our departmental histologist. Tissue sections were dewaxed in Histo-Clear (National Diagnostics, Atlanta, Georgia, US) for 2 x 5 min and rehydrated in a series of ethanol dilutions 100% (2 x 5 min), 90% (1 x 5 min), 70% (1x 3 min), 50% (1 x 5 min), followed by 5 min in PBS. Next, when tissues are fixed in cross-linking agents such as paraformaldehyde, these agents will covalently form cross-linked proteins, resulting a reduction in the available epitopes for antibody binding, therefore an antigen retrieval step is required. The tissue sections were submerged in Citrate, pH 6.0 buffer (A.MENARINI diagnostics, Wokingham, UK) and heated to 90-120°C in a pressure cooker for 20 min.

3.4.2 Streptavidin-Biotin Complex (ABC) Staining

Biotin, also known as vitamin H is a molecule with low molecular weight and is present in all living cells. In IHC, biotin is conjugated to antibodies or to the enzyme reporters use to detect target antigens. IHC staining intensity is a function of enzyme reporter activity and the sensitivity can be improved by amplifying the target antigen signal. The most common route for amplification is between tetravalent avidin (biotin-binding protein) and biotinylated antibodies (bound to the antigen). However, the carbohydrate content and basic isoelectric point of streptavidin results in high non-specific antibody binding (Bratthauer, 1994). Therefore, serum blocking is added to prevent non-specific binding before

3.4 Visual detection of proteins

the addition of primary and secondary antibodies. All incubation steps were carried out in a humidified chamber at RT and washing steps in PBS for 5 min.

After incubation with citrate buffer, pH 6.0 a wax pen was used to draw boundaries around the sections to prevent liquid flowing out. Next, 2% (v/v) blocking buffer (of the species in which the secondary antibody was raised) in PBS was added for 15-20 min. The blocking solution was removed by blotting before incubation with the primary antibodies (Table 3.2) for 1 h. Biotinylated secondary antibodies diluted in 2% (v/v) human AB serum were added for 30 min, followed by incubation with streptavidin-biotin peroxidase complex (ABC reagent, Vector Laboratories, Peterborough, UK) for 30 min. Next, the substrate 3'-diaminobenzidine (DAB, Sigma, St. Louis, Missouri, US), was added to the sections for between 1-5 min, where it was converted into an insoluble brown product by the enzyme peroxidase. Slides were counterstained in Carazzi's haematoxylin (Clin-Tech, Guildford, UK) for 5 min, rinsed in tap water and mounted in glycerol gelatin. Images were acquired using the Leica DC500 microscope (Leica, Wetzlar, Germany) and the Leica IM50 software.

3.4.3 Immunofluorescence on live cells

The purity of EVT cells isolated can range from 40-90%, determined by Immunofluorescence and flow cytometry. For migration experiments, the purity is usually determined by either fixing the cells post experiment and staining with an antibody for HLA-G or by taking a sample from the cell isolate for flow cytometric analysis. However, cell migration analysis is performed on all cells, irrespective of whether the cell is HLA-G +. Therefore, there is a need to identify EVT cells during imaging so that only cells that are HLA-G + are analysed. Antibodies that are conjugated with the fluorescence can be used without the need for fixation and secondary staining in live cell imaging.

HLA-G antibody (clone: G223, Bio-Rad, Langford, UK) was added to isolated EVT in 1:40 ratio and incubated at RT for 20 min. To prevent isolated cells from clumping together and reducing antigen-antibody binding sites, the cell solution was suspended in PBS without calcium and magnesium (Thermo Fisher Scientific, Waltham, MA, USA) and 1% v/v FCS. After incubation, the cell solution was

3.4 Visual detection of proteins

washed with EVT cell medium and kept out of direct light to reduce fluorescence bleaching.

3.4.4 Whole mount decidua staining

3.4.4.1 Staining protocol

A piece of decidua basalis was identified and using a razor blade, cut into a 1 cm x 1 cm piece. To remove blood, it was rested in RPMI medium supplemented with 10% FCS for 30 min in a 9 cm petri dish and spin rotated for a further 30 min while enclosed in an eppendorf tube. The tissue was then washed a further 5 times and fixed for 48 h at 4°C with 2% (v/v) PFA and 30% (w/v) sucrose (Sigma, St. Louis, Missouri, US), with both diluted with PBS. After fixation the tissue was washed in 30% (w/v) sucrose diluted in PBS for 2 h at RT. Primary antibodies (Table 3.2) of collagen I, II, III, IV and V (abcam, Cambridge, UK) and EPCAM (Cell signaling Danvers, Massachusetts, US) were added at dilutions of 1/100 and 1/50 respectively in PBS at RT for 1 h. Secondary antibodies (Table 3.3) from the same species as the primary antibody were incubated at RT for 1 h. Dapi hoechst (Thermo Fisher Scientific, Waltham, MA, USA), which stains for nuclei was added at a dilution of 1/10, 000 in PBS for 15 min at RT. The tissue was washed with 30% (w/v) sucrose diluted in PBS between all staining steps. The sample was stored at 4°C in PBS before imaging.

3.4.4.2 Imaging

The decidua sample was imaged using a ZEISS LSM 700 (Zeiss, Oberkochen, Germany) confocal microscope. It was mounted on an IBIDI 35 mm glass bottom dish (IBIDI, Planegg, Germany) and a circular glass slide was then placed on top and gently flattened using tweezers. Imaging was done at RT using a 10x and 40x objective lens. The images were exported as .lsm files with post-processing using FIJI (ImageJ, v. 2.0.0-rc-14/1.49 g) software.

3.4 Visual detection of proteins

Receptor	Clone	Species	Isotype	Tissue	Fluorophore /Label	Dilution	Incubation time (min)	Supplier
Primary Cytokeratin 7	OV-TL 12/30	Mouse	IgG1	Paraffin	na	1/150	30	Dako, Santa Clara, California, US
Primary HLA-G	MEMG1	Mouse	IgG1	Paraffin	na	1/30	60	Quantum Biosystems, Osaka, Japan
Primary HLA-G	G233	Mouse	IgG1	Live cells	FITC conjugated	1/40	20	Bio-Rad Langfor, UK
EPCAM	VU1D9	Mouse	IgG1	Whole mount	PE conjugated	1/50	60	Cell signaling, Danvers, Massachusetts, US
Collagen I,II,III,IV,V	na	Rabbit	IgG	Whole mount	na	1/100	60	Abcam, Cambridge, UK

Table 3.2: Primary antibodies used in protein visualisation.

3.4 Visual detection of proteins

Receptor	Fluorophore/label	Species	Dilution	Incubation time (min)	Supplier
Mouse IgG	Biotinylated	na	1/200	30	Vector Labs, Peterborough, UK
Rabbit IgG	Alexa Fluor 647	Goat	1/200	60	Abcam, Cambridge, UK

Table 3.3: Secondary antibodies used.

3.5 Flow cytometry

Isolated EVT cells were distributed at approximately 1×10^6 cells/well in 200 μ l of fluorescence-activated cell sorting (FACS) wash (FW, 1xPBS, 2% Fetal Calf Serum, 2mM EDTA). Cells were then stained for 1 h at 4°C in 30 μ l of cocktail. After staining LIVE/DEAD Aqua (ThermoFischer, Scientific, Waltham, MA, USA) was added for 30 min at 4°C, cells were fixed in 1% PFA for 10 min at RT. Finally, fixed cells were resuspended in FW ready for acquisition using a Fortessa (BD Biosciences, Franklin Lakes, New Jersey, US) flow cytometry instrument. To correct for spectral overlap during multi-colour flow cytometry experiments, colour compensation was performed with single colour stained beads. Data was analysed using FlowJo (10.1, version 7, FlowJo, Ashland, Oregon, US) software. The antibodies used in flow cytometric analysis are listed in Table 3.4.

Receptor	Clone	Fluorophore	Supplier
HLA-G	MEM-G/9	PE-Cy5	Abcam, Cambridge, UK
CD45	HI30	PE	Biolegend, San Diego, California, US

Table 3.4: Primary antibodies used in flow cytometry.

3.6 Atomic force microscopy

Atomic force microscopy (AFM) was used to determine sample elasticity when exposed to compressive forces via a cantilever probe. Samples were placed on an inverted optical microscope (Axio Observer.A1, Carl Zeiss, Oberkochen, Germany) and indentation measurements were taken with a JPK Nanowizard Cellhesion 200 AFM (JPK Instruments, Berlin, Germany). Tipless silicon cantilevers (Arrow-TL1, NanoSensors, Neuchatel Switzerland; and SICON-TL, Applied Nanostructures, Mountain View, California, US) with a spring constant of 0.01 to 0.1 N/m were used in these experiments. An estimate of the spring constant was calculated

3.6 Atomic force microscopy

via the thermal noise method (Hutter and Bechhoefer, 1993) and a polystyrene bead ($37.28 \pm 0.34 \mu\text{m}$ diameter; Microparticles GmbH, Berlin Germany) was glued onto each cantilever using ultraviolet curing glue (Loctite, Düsseldorf, Germany) prior to all measurements.

3.6.1 Sample preparation

3.6.1.1 Human tissue samples

Decidua samples were separated into suspected implantation and non-implantation sites and using a razor blade, cut into 1 cm x 1 cm pieces. For the placenta, a smaller cross section of 0.5 cm x 0.5 cm was cut. Non-pregnant endometrium biopsies were received in tubular form and had a diameter of approximately 0.25 cm. Samples were stored at 21°C in RPMI medium supplemented with 10% FCS. All samples were embedded on a 35 mm plastic dishes with the addition of 2.5% (w/v) agarose (stored at 40 °C, Sigma, St. Louis, Missouri, US) until only the edges were submerged, leaving only the centre exposed. For the placenta villi, half of the sample was embedded because villi are thin strands, which made it prone to detachment. Agarose takes 1 min to set as it cools. Once set, medium was added filling the dish, before measurement with the AFM. After measurements were complete, decidual and endometrium samples were fixed as described in 3.4.1. Decidua samples were stained with HLA-G to localise the sample to implantation or non-implantation site. Endometrium samples were stained with hematoxylin and eosin (H&E) by IHC to confirm presence of luminal surface epithelium.

3.6.1.2 Matrigel

The stiffness of five Matrigel batches (LOT: 6270006, 6221009, 6291008, 6214005 and 6277004) was determined using AFM. Matrigel was thawed by placing a 500 μl eppendorf tube on ice. Once thawed 20 μl drops were pipetted onto a 30 mm petri dish and placed at 37 °C for 20 min to set. Once set the dish was filled with EVT medium. The stiffness of Matrigel diluted to 80% (v/v), 60% (v/v) and 40% (v/v) with EVT medium was also measured.

3.6.1.3 Matrigel-alginate composites

Matrigel was thawed by placing a 500 ml eppendorf tube on 4 °C ice at RT. Sodium alginate (Sigma, St. Louis, Missouri, US) at a concentration of 25 mg/ml was dissolved in deionized (DI) water. The powder was added slowly on a magnetic stirring plate to prevent the solution clumping. Once dissolved the solution was autoclaved at 121 °C and 100 kPA for 15 minutes, then passed through a 0.22 µm filter. Calcium chloride (Sigma, St. Louis, Missouri, US) solution used to crosslink the alginate was dissolved to 1.22 M stock concentration with DI water. This stock solution was also passed through a 0.22 µm filter. All subsequent steps were carried out inside a laminar flow hood.

A solution of 46% Matrigel, 46% Alginate, 6% cell solution (for the AFM measurements, no cells were embedded, only HAMS-F12 medium) and 2% phenol red (for gel visualisation, Sigma, St. Louis, Missouri, US) was prepared in a 500 µl eppendorf tube on 4 °C ice. The solution was pipetted 60 times being careful not to generate air bubbles. A homogeneous red colour from the phenol red was a good indicator to whether the solution was well mixed. The composite gels were prepared in IBIDI-4 well plates (IBIDI, Planegg, Germany), which were used in the cell migration experiment. It was important the gels were prepared exactly as they were used in subsequent experiments. The IBIDI plates have 4 wells, each with a 10 µl volume. Each well was filled with 7 µl of the Matrigel-alginate solution and incubated at 37 °C for 20 min to enable Matrigel to set. Once set, 3 µl of the desired concentration of calcium chloride (0.6 M and 0.08 M in this study) was added to the wells. After 2 min, the calcium chloride solution was removed with a pipette and washed once quickly with PBS and then once more but this time it was left for 2 min to allow any excess calcium chloride to diffuse out. For AFM measurement, the plastic casing of the IBIDI wells was removed from the culture dish by gently twisting it clockwise. A pipette was used to push the gels out of the wells and into the well plates used for AFM measurements. The gels were then placed on 10 µl flat layer of Matrigel to prevent the gel from floating and incubated at 37 °C for 10 min. The dish was filled with HAMS-F12 medium before measurement with the AFM.

3.6.1.4 Indentation experiments

Petri dishes containing the samples to be analysed were placed on a motorised xy stage, which allowed movement of the sample relative to the AFM cantilever. A CCD camera (The Imaging Source GmbH, Bremen, Germany) was used to image and track the position of the cantilever above the sample. This setup was used to locate and define an area of interest on the sample on which AFM measurements were taken. A custom python script broke down this area into 20 x 20 μm squares, inside which a single measurement was taken. The motorised stage was moved as measurements were taken to perform a raster scan of the area of interest. Following analysis, the set of measurements taken were reconstructed to produce elastic modulus heat-maps.

In this study, the elastic properties are defined as an “apparent” elastic modulus, because assumptions of elastic contact do not fully apply for biological materials. Biological materials are inhomogeneous and absolutely inelastic. Apparent elastic modulus is therefore a measure of stiffness but not a true material property. For each apparent elastic modulus measurement, the cantilever probe was lowered onto the surface of the sample at a speed of 10 $\mu\text{m/s}$. As the probe made contact with the sample it produced an indentation at the time that it deformed. The probe continued to be lowered onto the sample until a force of 15 nN was reached. At this point the probe was retracted at a speed of 50 $\mu\text{m/s}$, the sample moved, and a measurement repeated at a different location. A threshold force of 10 nN on the cantilever-bead combination used translated to a maximum indentation depth δ into the sample of 1 to 5 μm at sample stiffnesses close to those of decidua.

The AFM carried out readings of cantilever z-position and cantilever angle (directly related to force applied). This produced force-distance curves for every indentation of the sample. Using a custom MATLAB (R2008a, Mathworks, Natick, Massachusetts) script a stiffness value was obtained from each of these curves using the Hertz model (Vinckier and Semenza, 1998). Contact of the polystyrene bead on the sample was modelled as a contact between a sphere and a half space, which may be used to calculate the apparent elastic modulus E .

3.6 Atomic force microscopy

$$F = \frac{4}{3}ER^{\frac{1}{2}}\delta^{\frac{3}{2}} \quad (3.1)$$

F being the force applied at the indentation depth δ chosen, and R the radius of the polystyrene bead. This relies on the assumptions that the stiffness of the polystyrene bead is much greater than that of the sample being measured. Polystyrene has an elastic modulus in the 10^9 Pa range, much higher than the 10^2 to 10^4 Pa range covered in these experiments.

For the Hertz model to be valid, strain applied to the sample must be small and indentation depth must be much smaller than the sample thickness. Apparent elastic modulus was calculated for an indentation depth δ of 2 μm , which resulted in a contact radius $a = \sqrt{R\delta}$ of approximately 8 μm . Biological materials tend to begin exhibiting deviations from the elastic behaviour at compressive strains $\epsilon = 0.2a/R > 0.02$, which places the measurements taken within an acceptable range. The condition $a/h < 0.1$, h being the thickness of the sample analysed, is also satisfied, which is necessary to treat the sample as a half space (Lin et al., 2009).

3.6.1.5 Data quantification and statistical analysis

A minimum of 30 measurements were taken for every biological replicate. As a quality control step, the resulting force-displacement curves were evaluated by hand and any failed measurements were excluded from further analysis. The median of all the measurements were taken to provide an apparent elastic modulus value for each biological replicate. Values were grouped together according to the type of tissue analysed and compared between groups using the Mann-Whitney, Wilcoxon signed-rank test and Kruskal-Wallis ANOVA with multiple comparison tests. Statistical analysis and data plotting was carried out using GraphPad Prism, Version 7 (GraphPad Software, Inc., La Jolla, Ca, US).

3.7 Extravillous trophoblast cell tracking

3.7.1 In Matrigel-alginate gels of varying stiffness

Matrigel-alginate gels were prepared as described in 3.6.1.3 to minimise variability in gel stiffness between AFM and cell experiments. EVT cell suspension was prepared as described in 3.2.1 and stained with HLA-G antibody to confirm presence of the extravillous phenotype. A concentration of 8×10^6 cells/ml in EVT cell medium was embedded in a solution comprising of 46% Matrigel, 46% Alginate, 6% cell solution and 2% phenol red in a 500 μ l eppendorf tube on ice. The gels were formed in IBDI 4-well dish, cross-linked with 0.6 M and 0.08 M calcium chloride. A volume of 200 μ l of EVT medium was added directly on top of the wells and 1.5 ml in the outer well of the dish, to prevent the gel dehydrating. The dish was incubated for 1.5 h at 37 °C before cell tracking.

3.7.1.1 Imaging

EVT motility was tracked using a ZEISS LSM 700 confocal microscope. The temperature of the environmental chamber enclosing the microscope was set to 37 °C, 1 h before cell tracking, to limit thermal drift during imaging. The HLA-G antibody and cell tracker dye were excited with a 488 and 555 laser respectively. An image was taken every 20 min for each stiffness condition for 2.3 h. Images with a length, width and height of 1278, 1278 and 121 μ m were taken with a 10x objective lens.

3.7.1.2 Analysis

Acquired images were exported as .lsm files with post processing using FIJI (ImageJ, v. 2.0.0-rc-14/1.49 g) software and the built-in plugin TrackMate (version 2.8.1). TrackMate allows the segmentation of spherical objects from a series of 3D images and to track these objects over time. In this study, only HLA-G+ cells were tracked with the blob (cell) diameter and image threshold set to 20 μ m and 60 respectively. The Laplacian of Gaussian (LoG) detector was used to detect each cell. The HyperStack Displayer and simple Linear Assignment Problem (LAP) tracker was chosen [Jaqaman et al. \(2008\)](#). A maximum linking distance

3.7 Extravillous trophoblast cell tracking

of 10 μm and a maximum gap-closing frame gap of 2 was chosen. Finally, a maximum gap-closing distance was 25 μm for trophoblast cells Oefner (2015). The analysis from TrackMate generated a spreadsheet, *spots in tracks statistics* containing Spot ID, x, y, and z coordinates, time frames and other variables. Data was exported to EXCEL (office 2016, Microsoft, Redmond, Washington, US) and the speed determined for the first 50 cells in each data set. speed was defined as the total distance travelled in the x, y, z coordinate divided by time.

3.7.2 In Matrigel and in a controlled oxygen environment

Primary EVT cells were tracked over 24 h in 1%, 8% and 21% oxygen concentration. EVT cell suspension was prepared as described in 3.2.1 and stained with HLA-G antibody to confirm presence of the extravillous phenotype. Fluorescent beads (FB) were added to the solution as reference points. One drop of OneComp eBeads (ThermoFischer, Scientific, Waltham, MA, USA) was added to a single well in a 96-well plate and reacts with antibodies of mouse, rat and hamster origin. A volume of 1 μl of Pacific Blue anti-human CD3 (BioLegend, San Diego, California, US) was added to the drop and incubated at RT for 20 min. This antibody was found to produce a clear signal when imaged under a confocal LSM microscope and was stable after overnight incubation at 37 $^{\circ}\text{C}$. The solution was washed three times in PBS and resuspended in EVT medium.

A concentration of 8×10^6 cells/ml in EVT cell medium was embedded in a solution comprising of 80% Matrigel, 10% cell solution and 10% FB solution. A 10 μl volume of Matrigel-cell-FB solution was pipetted into each of the four wells and incubated at 37 $^{\circ}\text{C}$ for 20 min for Matrigel to crosslink. A 200 μl volume of EVT medium was pipetted on top of the four wells and a further 1.5 ml in the outer ring of the dish, to keep the gel hydrated.

3.7.2.1 Imaging

EVT motility was tracked using a ZEISS LSM 700 confocal microscope. The temperature of the environmental chamber enclosing the microscope was set to 37 $^{\circ}\text{C}$, 1 h before cell tracking, to limit thermal drift during imaging. A 10x objective lens was used to image each well. The top and left corner of the well

3.7 Extravillous trophoblast cell tracking

was aligned with the top and left corner of the image. These well boundaries were used as alignment markers during subsequent imaging. The HLA-G antibody, cell tracker dye and FB were excited with a 488, 555 and 408 nm laser respectively. Each image had a length, width and height of 1278, 1278 and 805 μm respectively. Between 1-2 wells per culture dish were imaged, depending on the observed number of HLA-G+ cells. Images for the dish placed at 21% oxygen were taken first, followed by the 8% and 1% condition. After each condition had been imaged, the dishes were placed in incubators at the required oxygen conditions and at 5% CO_2 and 37 $^{\circ}\text{C}$. For the 1% condition, cells were placed at 15% and the oxygen concentration was gradually reduced every hour to 10%, 5% and finally 1% in order to reduce stress to cells. After a 24 h incubation, the wells were immediately imaged with the well boundaries used to align the image. All images were saved as .LSM files.

3.7.2.2 Analysis: coherent point drift

Coherent point drift (CPD) algorithm was used to find the spatial transformation of the FB between the images at 0 h and 24 h. The CPD algorithm was run using python programming language (version 3.5). The CPD algorithm was developed by (Myronenko and Song, 2009), with the version used in this thesis by (Wolfahrt, 2017).

Acquired images were exported as .lsm files and opened using FIJI software. The FB channel was separated from the cell tracker and HLA-G antibody channel. Each image was converted into an 8-bit greyscale image and saved as a .tif file. Blob detection algorithm, which is used to identify the FB and assign coordinates was obtained at (Wolfahrt, 2017). The command prompts for running the blob detection and CPD algorithm on example images are detailed in Appendix A.

The output of the CPD algorithm was a vector in a .mat file (Matlab, version R2014a, Mathworks, Natick, MA, USA) which was multiplied by the cell coordinates at 24 h. Cell coordinates (HLA-G stain) for images at 0 h and 24 h were determined using FIJI software and the built-in plugin TrackMate and described in full in 3.7.1.2. The cell coordinates at 24 h is now corrected for any shift in the image. The RMSD was determined as described in 5.3.3.2.

3.8 Microfluidic-based oxygen gradient assay

There are three parts to the set up of a microfluidic-based oxygen gradient assay: (1) microfluidic device fabrication (2) gas-liquid diffusion system assembly and (3) validation of oxygen throughout the system.

3.8.1 Microfluidic device fabrication

Device design. Microfluidic devices were fabricated using Polydimethylsiloxane (PDMS, Corning, Midland, Michigan, United States) polymer. Two microfluidic designs were used in this thesis with the channels separated by either: (1) 36 trapezoidal posts spaced 100 μm apart or (2) a 80 μm PDMS wall. Microfluidic devices were cast using silicon wafers. The trapezoidal based wafer was manufactured by Stanford Microfluidics Foundry (Stanford, California, US) and had been used previously ([Abbas et al., 2017](#)), whereas the PDMS wall based device was designed using AutoCAD (AUTODESK, Mill Valley, California, US) and manufactured by microLIQUID (microLIQUID, Arrasate, Spain). Each of the channels are accessible through two ports and have a length, width and height of 20,300 μm x 1, 300 μm and 150 μm respectively (Figure 6.5).

PDMS casting. Before use, the wafers were surface treated with 100 μl trichloro (1H,1H,2H,2H-perfluorooctyl) silane (Sigma, St. Louis, Missouri, US) for 24 h in a vacuum sealed desiccator, to enable easy peeling of PDMS. A 10:1 ratio of base to curing agent was mixed and degassed using a vacuum desiccator. PDMS was poured over the wafer and cured for 1 h at 80 $^{\circ}\text{C}$. The solid PDMS devices were cut and peeled from the wafer. Biopsy punchers (Selles Medical, Hull, UK) were used to create ports for the central (1 mm outside diameter) and two side channels (4 mm outside diameter).

Device bonding. Microfluidic devices were cleaned by removing dirt using adhesive tape (Scotch tape, 3M, Maplewood, Minnesota, US) and sterilized by washing in DI water and autoclaving at 121 $^{\circ}\text{C}$ and 100 kPA for 15 min. Before bonding with glass, glass slides were cleaned with ethanol and microscope grade paper (ThermoFisher Scientific, Waltham, MA, USA). The glass slide and PDMS

3.9 Microfluidic oxygen gradient system

device were placed on a petri dish and enclosed in a plasma cleaner (Harrick Plasma, Ithaca, NY, US) at high power for 1 min under vacuum. The PDMS and glass are oxidized forming bridge Si-O-Si bonds at the interface, creating an irreversible seal. To increase the strength of the seal, the two layers were pressed against each other for 30 s and placed at 80 °C for 15 min.

3.9 Microfluidic oxygen gradient system

3.9.1 Gas-liquid diffusion system

3.9.1.1 Glass sealed vessel

A 500 ml Kilner jar (Lakeland, Windermere, UK) was adapted by replacing its original cap with a brass plate (6.5 cm diameter, thickness: 1 mm). Four holes were drilled (5 cm apart, 1.2 mm drill bit) and hypodermic needles were soldered (outside diameter: 1 mm) to make ports. To seal the vessel a rubber o-ring (outside diameter (OD): 6.5 cm, inside diameter (ID): 4.5 cm) was cut from a sheet of rubber (thickness: 4 mm) and high vacuum silicone grease (Corning, Corning, NY, US) applied to the rim of the vessel. The original brass cap that came with the Kilner jar, was used to seal the brass plate and rubber o-ring to the jar (Figure 6.6).

3.9.1.2 Tubing

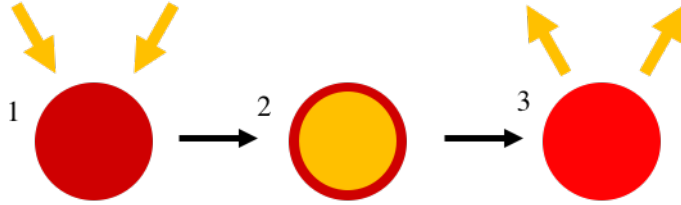
A 10 m length of gas-permeable platinum cured silicone tubing (OD: 1.81 mm, ID: 0.8 mm, Silex LTD, Lindford, UK) was used for gas-liquid diffusion inside the glass sealed vessel. Viton tubing (OD: 1.81 mm, ID: 0.8 mm, Cole-parmer, Vernon Hills, Illinois, US) of varying length (0.5 m from syringe pump to the vessel, 0.5 m from the vessel to the device and 0.3 m from the device to a waste container) was used as the gas impermeable tubing in this study. A syringe pump (Fusion 700, Chemyx, Stafford, Texas, US) was used to drive fluid flow.

3.9.2 Oxygen sensing

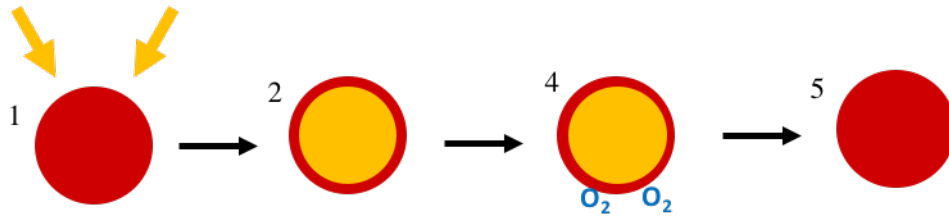
3.9.2.1 Real-time monitoring

For real-time monitoring a WITROX (Loligo Systems, Viborg, Denmark) 4-channel fluorescent based oxygen instrument was used. The principle is based on the quenching of luminescence by molecular oxygen and the concentration of oxygen is proportional to the amount of quenching (Figure 3.1). Dye molecules absorb light at their maximum absorption wavelength and exhibit long excited state life times and emit light at longer wavelengths. In the presence of oxygen molecules, the excited state of the dye is quenched by an energy transfer. The result is a reduction in the intensity of the luminescence of the dye and the oxygen concentration is proportional to the amount of quenching (Quaranta et al., 2012). The instruments light-emitting diode (LED) emit blue-green light, which travels through a fibre optic cable that excite oxygen sensitive dye coated within the sensors (optode).

No oxygen



Oxygen present



- | | |
|------------------------|--|
| 1. Absorption of light | 4. Energy transfer by collision of oxygen molecules |
| 2. Excited state | 5. Dye is quenched: emission of light is proportional to amount of quenching |
| 3. Emission of light | |

Figure 3.1: **Principle of optical oxygen sensor**

Concentration of gaseous oxygen in the vessel was measured using a 5 mm diameter sensor spot (PreSens, Regensburg, Germany) containing fluorescent dye attached to the inside of the vessel using silicone glue. The fibre optic cable was held in place and aligned facing the sensor spot using a polyphenylene ether (PPE) machined holder. For dissolved oxygen measurement at the inlet and outlet of the device, a flow-through sensor (Loligo Systems, Viborg, Denmark) was connected to tubing 5 cm from the microfluidic ports. The oxygen concentration was measured every second and was logged wirelessly using Bluetooth.

Sensor calibration. The sensors were calibrated before each experiment. For each sensor connected, a 2-point calibration in an oxygen free environment and air saturated environment was needed to convert sensor phase [°] values into

3.9 Microfluidic oxygen gradient system

oxygen units. Since the sensor phase [°] signal is temperature dependent, each channel was paired to a temperature input for the software to compensate oxygen values in real-time. An oxygen free environment was generated by either bubbling nitrogen gas (Genie, BOC, Guildford, UK) through DI water or the reaction of 20 mg/ml of sodium sulfite, (Sigma, St. Louis, Missouri, US) an oxygen scavenger and DI water. Nitrogen bubbling was preferred given the increased risk of fouling by precipitate forming on the sensor. However, this was not possible for the flow-through sensor as the rate of oxygen diffusion from atmosphere into DI water was faster than the rate at which nitrogen enriched DI water could be pumped through the sensor. An air-saturated environment was generated by bubbling air using an air pump (Amazon, Seattle, Washington, US) attached to an air stone. The solution was stirred so that oxygen gradients do not form in the water. Information on the sensor measurement specifications are listed in Table 3.5.

	Sensor spot	Flow-through sensor
Application	Gaseous O ₂ concentration in vessel	Dissolved O ₂ concentration at MF inlet and outlet
Measurement range	0-100% O ₂	0-100% O ₂
Limit of detection	0.03% O ₂	0.03% O ₂
Resolution	± 0.01 % O ₂ at 0.21 % O ₂ ± 0.1 % O ₂ at 20.9 % O ₂	± 0.01 % O ₂ at 0.21 % O ₂ ± 0.1 % O ₂ at 20.9 % O ₂
Accuracy	± 0.4 % O ₂ at 20.9 % O ₂ ± 0.05 % O ₂ at 0.2 % O ₂	± 0.4 % O ₂ at 20.9 % O ₂ ± 0.05 % O ₂ at 0.2 % O ₂
Precision	± 0.105 % O ₂	± 0.105 % O ₂
Sterilization process	Steam sterilization	Steam sterilization
Calibration	Two point calibration in O ₂ free environment (nitrogen gas) and air-saturated environment	Two point calibration in O ₂ free environment (sodium sulfite) and air-saturated environment

Table 3.5: Summary of real-time oxygen sensor specifications.

3.9 Microfluidic oxygen gradient system

3.9.2.2 Thin film sensor

Chemical make up of sensor solution The sensor solution was made up of toluene (Sigma, St. Louis, Missouri, US), polystyrene (Sigma, St. Louis, Missouri, US) and 5,10,15,20-Tetrakis(pentafluorophenyl)-21H,23H-porphine palladium(II) (PtTFPP) (Sigma, St. Louis, Missouri, US). Sensor solution was made up in 8 ml of toluene, adding 354 mg of polystyrene slowly while stirring and left for 24 h in a fume hood. PtTFPP dye (3.62 mg) was added and the sensor solution was sonicated for 30 s (Cole-parmer, Vernon Hills, Illinois, US). Sensor solution was made in batches, stored in the dark, and used within one week.

Knife coating sensor solution: The film was applied using a bar film applicator (BYK-Gardner, Wesel, Germany) to knife coat a thin layer on a glass slide (Figure 6.11). The glass slide was cleaned by application of ethanol and 5 min exposure to plasma. The bar film applicator was cleaned with ethanol before each use. A 200 μ l volume of sensor solution was pipetted on to one end of a glass slide, then pressed down using the applicator and the film was applied by moving the applicator in a fast motion along the slide.

Spin coating sensor solution: A glass slide was secured onto a metallic chuck that attaches onto the spin coater. A 200 μ l volume of sensor solution was pipetted on the centre of the glass slide and spin coated (MTI Corporation, St, Richmond, California, US) at 2500 revolutions per minute (rpm) for 5 min. Both coating techniques were carried out under a fume hood and left over night in the dark before integration with the microfluidic device.

Integrating thin film sensor to the microfluidic device. A layer of PDMS was spin coated onto the thin film sensor to enable bonding with the microfluidic device. Approximately 1 ml of a 10:1 ratio of base to curing agent was spin coated onto the glass-sensor layer for 5 min at 2500 rpm. This results in a layer of PDMS that is approximately 5 μ m thick (Tan et al., 2008). The PDMS was cured at 80 °C for 1 h. Clean microfluidic device, were prepared as described in 3.8.1 and plasma bonded, at high power to the glass-sensor-PDMS layer for 1 min under

3.9 Microfluidic oxygen gradient system

vacuum. The device was pressed down for 30 s and incubated at 80 °C for 15 min to increase the strength of the bond.

Measuring the thickness of the thin film sensor. The thickness of the thin film sensor was determined using a surface profiler (Taylor Hobson, Leicester, UK). A section of the film was sliced using a razor blade and a stylus (2 μm radius) was programmed to move across and over the edge of the film down to the glass surface. The step change from film to glass was used to determine the thickness of the film.

Imaging the thin film sensor: The sensor was imaged with a fluorescent microscope (Zeiss, Oberkochen, Germany), excited at 405 nm with emission at 650 nm. The focus dial was used to find the sensor layer and imaging was carried out at its maximum intensity.

3.9.3 Oxygen gradient generation

Filling vessel with N₂ gas. The vessel was sealed by application of vacuum grease onto the rim of the vessel and closed tight. Viton tubing was used to tie a knot on two of the four ports. N₂ gas was bubbled at a fixed flow rate of 12.5 l/min through an air tight plastic container (Lakeland, Windermere, UK) filled with 500 ml of DI water (6.7A). The last of the four ports was used as a vent. The oxygen concentration in the vessel was monitored in real-time using the sensor spot attached to the inside of the vessel. When the oxygen concentration reached 0%, the gas feed was switched off and viton tubing was used to tie a knot in the remaining ports.

Gel filling the device. Matrigel, collagen-1 (BD Biosciences, Franklin Lakes, New Jersey, US) and agarose gels were used in this study. Matrigel was used at concentrations between 50-100%, diluted in EVT medium. Collagen-1 was used to make a 1:1 composite with Matrigel. Agarose gel at 0.5% w/v was embedded in the central channel because it was able to withstand higher flow rates than Matrigel and it is inexpensive. Agarose was warmed in a microwave at 500 watts

3.10 Statistical analysis

for 1 min and carefully pipetted in the central channel of the device and placed at 4 °C for 1 h for the gel to set. For Matrigel and collagen-1, the gels were set by incubation at 37 °C for 20 min.

Assembly of oxygen gradient generation system. To generate an oxygen gradient, two gas sealed vessels were used, one for each side channel. Plastic syringes (BD Biosciences, Franklin Lakes, New Jersey, US) of volumes ranging between 10-60 ml were filled with DI water and pumped through the glass sealed vessels. The flow rates in this study ranged from 0.12 ml/hr to 54 ml/hr. The oxygen concentration in the vessel, and the dissolved oxygen at the inlet and outlet of one side channels was monitored in real-time using the witrox oxygen transmitter. Gas impermeable tubing was used to connect the vessels and the devices, as described in 3.9.1.2. For visualising the oxygen gradient, the system was assembled around a fluorescent microscope and imaged, as described in 3.9.2.2.

3.10 Statistical analysis

Data was analysed using GraphPad Prism, Version 7 (GraphPad Software, Inc., La Jolla, Ca, US). The normality of all data sets were tested using the D'Agostino & Pearson test. If the data was found not to follow a gaussian distribution, non-parametric tests were used to determine statistical significance. Tissue stiffness data was analysed using the Mann-Whitney, Wilcoxon signed-rank test and Kruskal-Wallis ANOVA with multiple comparison tests. Analysis of EVT migration under substrates of varying stiffness was carried out using the Mann-Whitney test. RMSD of EVT cells under varying oxygen concentration was analysed using the Friedman and Multiple Coparison test. Data sets that were found to be normal were analysed either using the paired or unpaired t-test. The p -values, a measure of statistical significance is given in each figure caption. Significance was set as $p < 0.05$.

Chapter 4

Tissue stiffness at the maternal-fetal interface

This chapter presents the results of experiments performed using atomic force microscopy (AFM) to determine tissue stiffness of human non-pregnant endometrium, pregnant endometrium (first trimester, decidua) and the placenta (first trimester). The migratory behaviour of extravillous trophoblast cells on three-dimensional substrates of different stiffness is investigated. Measurements taken with the AFM was done in collaboration with Mr Alejandro Carnicer in Dr Kristian Franze's group at the Department of Physiology, Development and Neuroscience at the University of Cambridge. Analysis of the raw AFM data was carried out by Mr Alejandro Carnicer.

4.1 Introduction

Biological tissues are composed of materials that transmit mechanical forces, which are known to regulate cell fate and behaviour. In recent years there has been renewed interest in the role of mechanics during embryonic development, adult stem cell potency and cancer cell invasion (Maître et al., 2016; Ahmed and French-Constant, 2016; Pathak and Kumar, 2012). Early in development, self-organisation of the embryo and maturation of the germ layers are known to rely on intrinsic mechanical forces and external mechanical input from the fluid surrounding the embryo (Vining and Mooney, 2017; Maître et al., 2016). As development progresses, extracellular matrix (ECM) content increases and intrinsic forces exerted by cells transition from cell-cell to cell-ECM (Cosgrove et al., 2016). Mechanical forces are thought to direct cell fate during organogenesis as progenitor cells differentiate into diverse specialised functions in fetal organs. Seminal experiments *in vitro* by Engler et al., demonstrated the importance of mechanics in directing adult stem cell fate (Engler et al., 2006). Human mesenchymal stem cells (hMSCs) were placed in culture on soft matrices that mimic brain, stiffer matrices that mimic muscle and rigid matrices that mimic bone. Reprogramming of these lineages specified by the matrix elasticity occurred after several weeks of culture. From this work, there has been an effort to design synthetic matrices with defined mechanical properties, that direct stem cell differentiation for tissue regeneration applications (Gjorevski et al., 2016).

The importance of mechanics in cell fate and behaviour has led to the characterisation of most tissues and organs in the human body (Vining and Mooney, 2017; Cox and Erler, 2011). One area in which mechanics has been largely overlooked is pregnancy. Specifically, implantation of the embryo into the endometrium and development of the placenta. The first aim of this chapter, is to use atomic force microscopy (AFM) to determine tissue stiffness of the non-pregnant endometrium, pregnant endometrium (decidua) and first trimester placenta.

4.1.1 Potential implications of tissue stiffness on trophoblast cell migration

There are questions as to whether tissue stiffness is one of the regulators of trophoblast invasion. Stiffness is known to regulate cell migration with *in vitro* studies showing cells preferentially migrate from less towards stiffer substrates, a process termed durotaxis (Isenberg et al., 2009; Lange and Fabry, 2013). The mechanism of cell migration *in vivo* is more complex than current lab based studies with the addition of metalloproteinases, migration in a 3D microenvironment and a complex architecture of ECM proteins. There has been no comprehensive study investigating stiffness as a regulator of EVT invasion. The second aim of this chapter, is to ask whether substrate stiffness affects EVT cell migration *in vitro*. An interpenetrating network of Matrigel-alginate gel is fabricated, which allows control of stiffness independent of ECM protein concentration and substrate pore size (Chaudhuri et al., 2014). Matrigel is a reconstituted basement membrane and is approximately 60% laminin, 30% collagen IV, and 8% entactin (Corning, 2012). It is the primary 3D gel used to study EVT cell behaviour *in vitro* (Onogi et al., 2011).

4.1.2 Extracellular matrix at the maternal-fetal interface

During the menstrual cycle, production of the hormone progesterone drives changes in the lining of the uterus (Aplin and Jones, 1989; Ruiz-Alonso et al., 2012). The endometrium undergoes decidualization, which is characterised by the proliferation and differentiation of endometrial stromal cells into specialised secretory decidual cells (Benirschke et al., 2012). Progesterone production regulates ECM in the decidua, up-regulating collagen IV, laminin and down-regulating collagen VI (Oefner et al., 2015). The decidua throughout pregnancy is rich in laminins, collagens, heparan sulfate proteoglycan and fibronectins (Burrows et al., 1996). Collagens are the main structural proteins of the ECM and are classed as either fibrillar (e.g. collagens I-III, V and XI) or non-fibrillar (e.g. collagens IV, VI) (Gjaltema and Bank, 2017). Production of ECM proteins is not limited to decidual cells, with strong evidence that invading EVT deposit collagen IV (Oefner et al., 2015). In the first trimester placenta, collagens I, IV, fibronectin

4.2 Atomic force microscopy

and fibrillin-1 are abundant (Aplin and Jones, 1989). It is important to stress, that the local mechanical microenvironment surrounding a cell is interlinked with the type, abundance and distribution of the ECM. Therefore, when considering the implications of mechanics on cell fate and behaviour, it is important to also consider ECM proteins.

There is a need for better characterisation of ECM composition, spatial distribution and organisation in the decidua to provide a more complete understanding of its role in normal and pathological tissues. In recent years, decellularization techniques have enabled high resolution imaging of ECM in whole animal organs (Mayorca-Guiliani et al., 2017). However, these techniques are complex, long and remove cells, which prevents understanding of the localisation of the ECM. The third aim of this chapter, is to image the architecture of collagen in the decidua, without decellularization, using simple staining techniques and importantly, in 3D. Whole mount staining was used, which is the staining of small pieces of tissues without sectioning and is a common technique in embryo and zebrafish research.

4.2 Atomic force microscopy

There are number of methods that can be used to probe stiffness of biological tissues, which include atomic force microscopy (AFM), infrared laser traps (optical tweezers), magnetic tweezers, indentation and traction force microscopy (TFM) (Vining and Mooney, 2017). AFM was used in this study and it has several advantages over other measuring techniques. AFM can be used to obtain a high resolution image at the nanoscale, and can be used in aqueous environments, preserving biological tissue *in vitro* (Last et al., 2010).

AFMs operate by measuring the force necessary for a cantilever to indent into a sample, in a feedback mechanism, as it scans a surface (Figure 4.1). A laser beam is used to detect cantilever deflections, by reflecting an incident beam off the cantilever. A position sensitive photo diode (PSPD) is used to detect changes in the direction of the reflected beam. The deformation of the cantilever is given as a function of the indentation depth of the tip into the tissue sample. The curve from this relationship can be fitted with the Hertz model (Vinckier and

4.2 Atomic force microscopy

(Semenza, 1998) for an estimation of the apparent elastic modulus of the sample at the indentation spot. Apparent elastic modulus is a measure of the stiffness of a solid object and is used because the assumptions of elastic contact do not fully apply for biological materials. (Last et al., 2010; Collinsworth et al., 2002).

Careful attention is needed when selecting an appropriate cantilever for sample measurement. Optimal force measurements by the cantilever should result in a large amount of cantilever deformation, together with a marked degree of sample indentation. To achieve this, cantilevers that match the stiffness of the samples measured need to be used (Gavara, 2017).

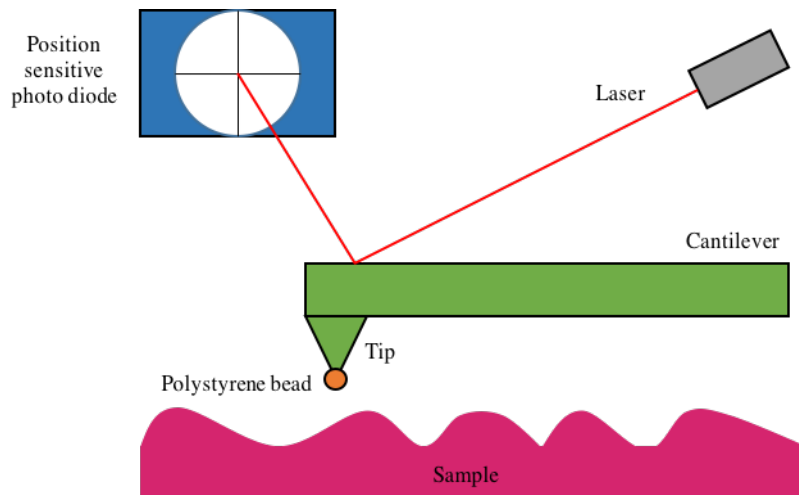


Figure 4.1: **Basic concept of AFM.** AFM is used to determine the stiffness of samples by measuring the force between a cantilever and the sample in a feedback mechanism. The reflected laser beam strikes a position sensitive photo diode consisting of a four segment photo detector and this is used to determine the angular deflections of the cantilever. The Hertz model can be used to estimate the apparent elastic modulus.

4.3 Samples studied

4.3.1 Human tissue

Decidua samples at gestational ages between 8-10 weeks were obtained from surgical terminations of pregnancy (Table 4.1). The full protocol for tissue isolation is detailed in the materials and methods, chapter 3.6.1.1. The decidua was separated into suspected implantation and non-implantation sites by eye. Implantation sites were identified with the presence of placental villi attached to the decidual tissue. Each sample was fixed after AFM testing and stained with HLA-G antibody by immunohistochemistry (IHC). A positive staining confirmed the presence of EVT and the implantation site was now termed decidua basalis, whereas those with negative staining was termed decidua parietalis. Six of the seven decidua samples are matched and originate from the same patient. Placenta villi were separated from the decidua and is easily identified by eye. All samples were stored in Roswell Park Memorial Institute (RPMI) medium, with fetal calf serum (FCS) and tested within ten hours of receipt.

Decidua Basalis Sample no.	Gestational age (weeks + days)	Decidua Parietalis Sample no.	Gestational age (weeks + days)	Placenta Sample no.	Gestational age (weeks + days)
P062	9 + 0	P062	9	P0653	8+0
P064	8+5	P064	8+5	R006	9+5
P065	8+4	P065	8+4	R008	9+2
R006	9+5	R006	9+5	R015	8 + 0
R007	9 + 0	R007	9	R016	8+4
R008	9+2	R008	9+2		

Table 4.1: Human decidua and placenta samples.

Non-pregnant endometrium was obtained from biopsies on women with infertility issues (between 2-5 miscarriages) (Table 4.2). The samples were taken between 7-11 days past peak luteinizing hormone (LH) production, indicating the

4.3 Samples studied

women were in the secretory phase of the cycle. All samples were stored in RPMI medium, supplemented with FCS and tested within eight hours of receipt. Each sample was fixed and stained with hematoxylin and eosin (H & E) by IHC to confirm presence of luminal epithelium, the surface of the endometrium.

Endometrium sample no.	Days past LH peak	No. of miscarriages in first trimester
217	11	3
218	11	4
219	11	4
221	10	5
222	7	2

Table 4.2: Human non-pregnant endometrium samples.

4.3.2 Hydrogels

The primary function of EVT cells is to invade the decidua and remodel the maternal spiral arteries. In this study, the aim was to conduct an experiment to determine whether stiffness regulates trophoblast migration in: (1) a substrate of physiologically relevant stiffness, (2) in 3D, and (3) a substrate whereby stiffness can be controlled independent of ECM concentration and pore size. These requirements were considered for Matrigel and an interpenetrating network of Matrigel and alginate.

Matrigel is the primary gel used to culture trophoblast cells in 3D, for both stem cell and cell migration studies. The stiffness of five different Matrigel batches was tested to determine whether Matrigel is mechanically relevant to the decidua, and if it could be used to modulate stiffness. An interpenetrating network of Matrigel and alginate was fabricated, based on the published protocol by Chaudhuri *et al.*, (Chaudhuri *et al.*, 2014). Alginate is a polysaccharide derived from seaweed, composed of mannuronic (M) and guluronic (G) acid and presents no intrinsic cell-adhesion ligands. Only the G-blocks of alginate are believed to participate in intermolecular cross-linking with divalent cations (e.g. Ca^{2+}) to form hydrogels

(Rinaudo, 2014). Stiffness can be tuned by changing the concentration of Ca^{2+} alone while ECM content in Matrigel stays the same. Importantly, Chaudhuri *et al.*, conduct experiments that show pore size and accessibility of ligand binding sites remain constant (Chaudhuri *et al.*, 2014). This is because the addition of Ca^{2+} only further bridges the G blocks already cross-linked.

4.4 Results

4.4.1 Tissue stiffness

4.4.1.1 Decidual tissue stiffness

Decidual samples were embedded in 2.5% (w/v) agarose and submerged in RPMI medium, supplemented with FCS in 35 mm culture plates. A CCD camera was used to image and track the position of the cantilever above the sample. Initial measurements were taken to estimate the stiffness of the samples, in order to choose an appropriate cantilever. A ‘medium’ cantilever (0.1 N/m) was chosen for decidua basalis samples and a ‘soft’ cantilever (0.01 N/m) for decidua parietalis. In addition, because these samples are soft a round polystyrene bead was glued onto the tip. For every sample at least 30 measurements were taken, with each measurement in a $20\text{ }\mu\text{m} \times 20\text{ }\mu\text{m}$ square. As a quality control step, the resulting force-displacement curves were evaluated by hand and any failed measurements were excluded from further analysis (for full detail on AFM indentation see materials and methods, chapter 3.6.1.4).

The median of these measurements was taken to give a value for apparent elastic modulus. The apparent elastic modulus for all samples was lower for decidua parietalis compared to basalis (Figure 4.2A). The data was found not to follow a gaussian distribution using the D’Agostino & Pearson normality test. This result was found for all tissue samples measured in this chapter. Consequently, statistical analysis was conducted using non-parametric tests. The apparent elastic modulus of the decidua basalis and decidua parietalis was 651.9 Pa and 191.6 Pa respectively ($P = 0.0312$, Wilcoxon signed-rank test, $N = 6$; Figure 4.2B).

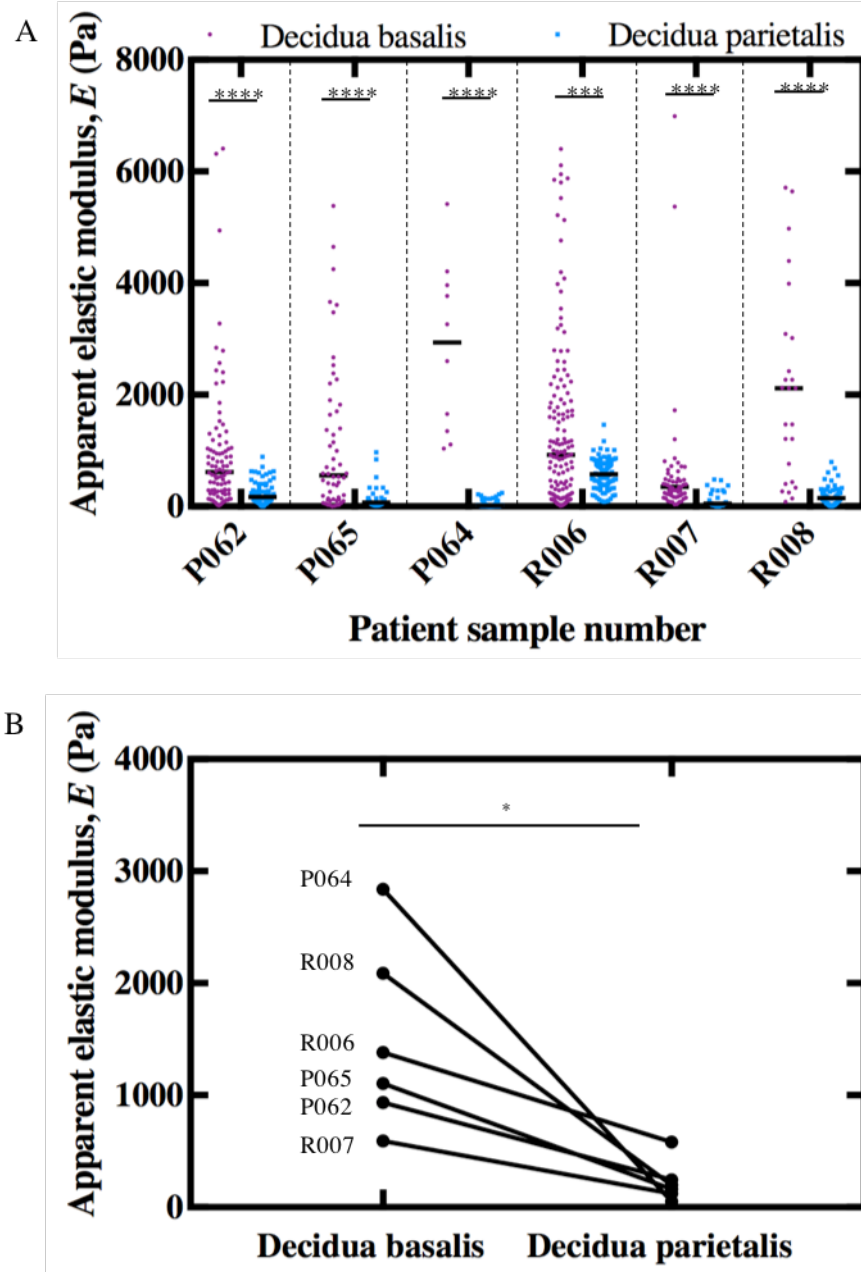


Figure 4.2: **Stiffness of human decidua.** Decidua was separated into suspected implantation and non-implantation sites and the tissue stiffness was measured using an AFM. (A) A plot of the individual data points for each sample (Mann-Whitney, $***P < 0.0001$, $***P < 0.001$, $n=10-148$). (B) Comparison of each patient matched sample (Wilcoxon signed-rank test, $*P < 0.05$, $N = 6$).

4.4 Results

Decidua basalis exhibited regions of increased stiffness, resulting in a wider distribution of the apparent elastic modulus measured. 140 of the 386 measurements were over 1 kPa accross the six patient samples for basalis compared to 6 out of 362 in parietalis. Regions of increased stiffness can be seen in the stiffness maps, where each square has dimensions of 20 μm x 20 μm and crosses represent failed measurements (Figure 4.3). Failed measurements are common during AFM testing of biological specimens, especially in soft samples. These can be caused by an uneven sample height resulting in differences in sample indentation depth, sensitivity of the cantilever to vibrations in soft samples and sticking of the tip to the sample resulting in a slower retraction speed. In figure 4.3, regions of increased stiffness are localised and not homogenous through out the tissue. It is not possible to know what exactly the AFM cantilever is measuring and therefore what is contributing to the increase in stiffness in each of the decidua basalis samples. Given that these samples are matched from the same patient, measured at the same time after the surgical termination with identical sample preparation, this suggests differences in the mechanical microenvrionment within the decidua.

After the stiffness of each sample was measured, it was removed from agarose, fixed and embedded in paraffin wax for tissue sectioning. The surface that was measured with the AFM was the same as that sectioned and fixed onto a glass slide. A representative image from immunohistochemistry staining from sample R008 is shown in Figure 4.4. The sections were stained for HLA-G (Figure 4.4A) and cytokeratin-7 (Figure 4.4B), which also stains epithelial cells around the glands, to confirm presence of the extravillous phenotype and therefore decidua basalis was measured. Similarly, for decidua parietalis, an image from sample R008 is shown in Figure 4.5. It is negative for HLA-G (Figure 4.5A) and only the glands stain positive for cytokeratin 7 (Figure 4.5B). Both samples stain negative for the isotype control (Figure 4.4C & 4.5C).

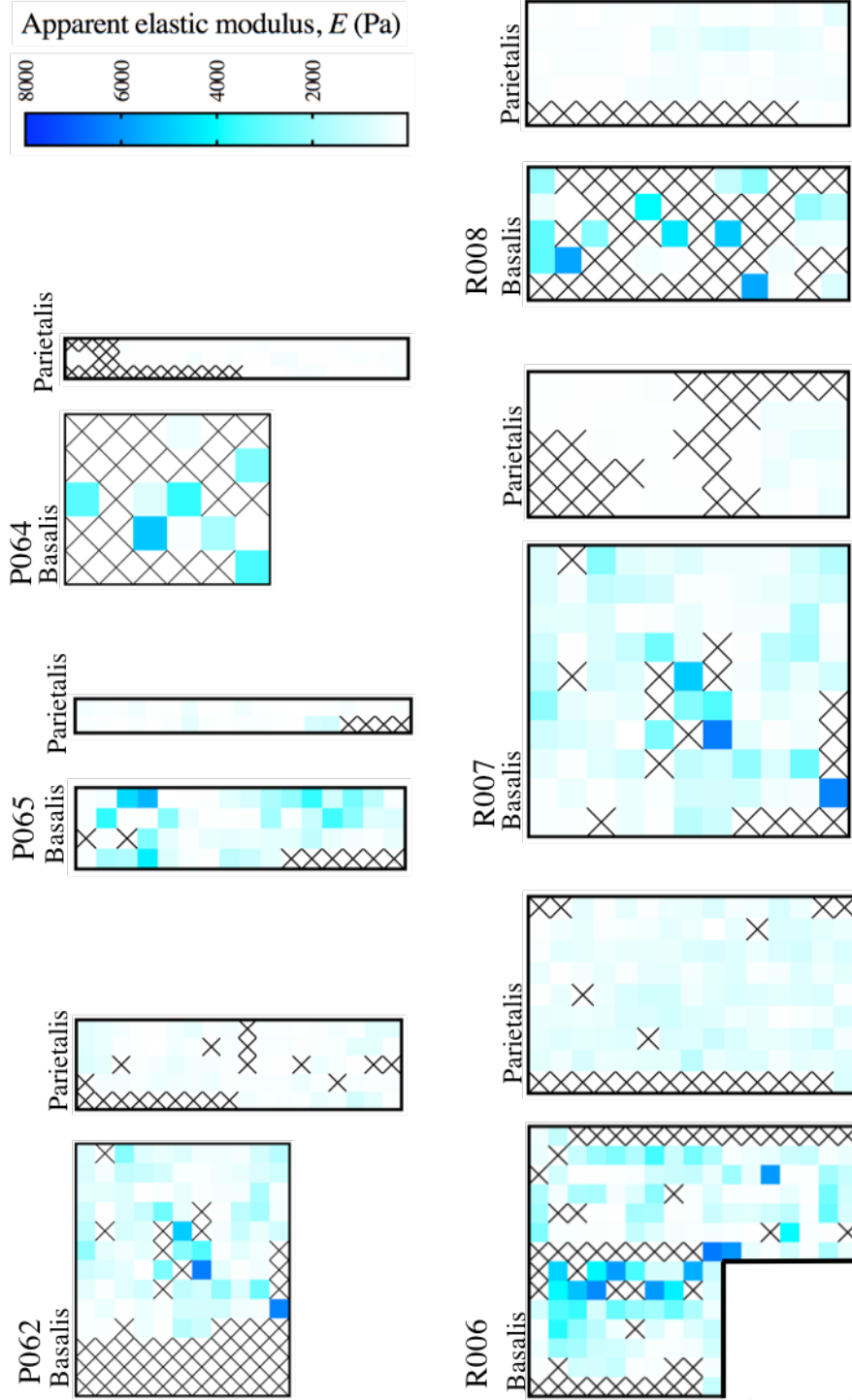


Figure 4.3: Map of apparent elastic modulus values for decidua basalis and parietalis. Each square has an area of $20 \mu\text{m} \times 20 \mu\text{m}$, inside which a single measurement was taken. A motorised stage is moved to perform a raster scan of the area of interest. Crosses mark failed measurements, which are determined by evaluating the force-displacement curves by hand during post experiment analysis.

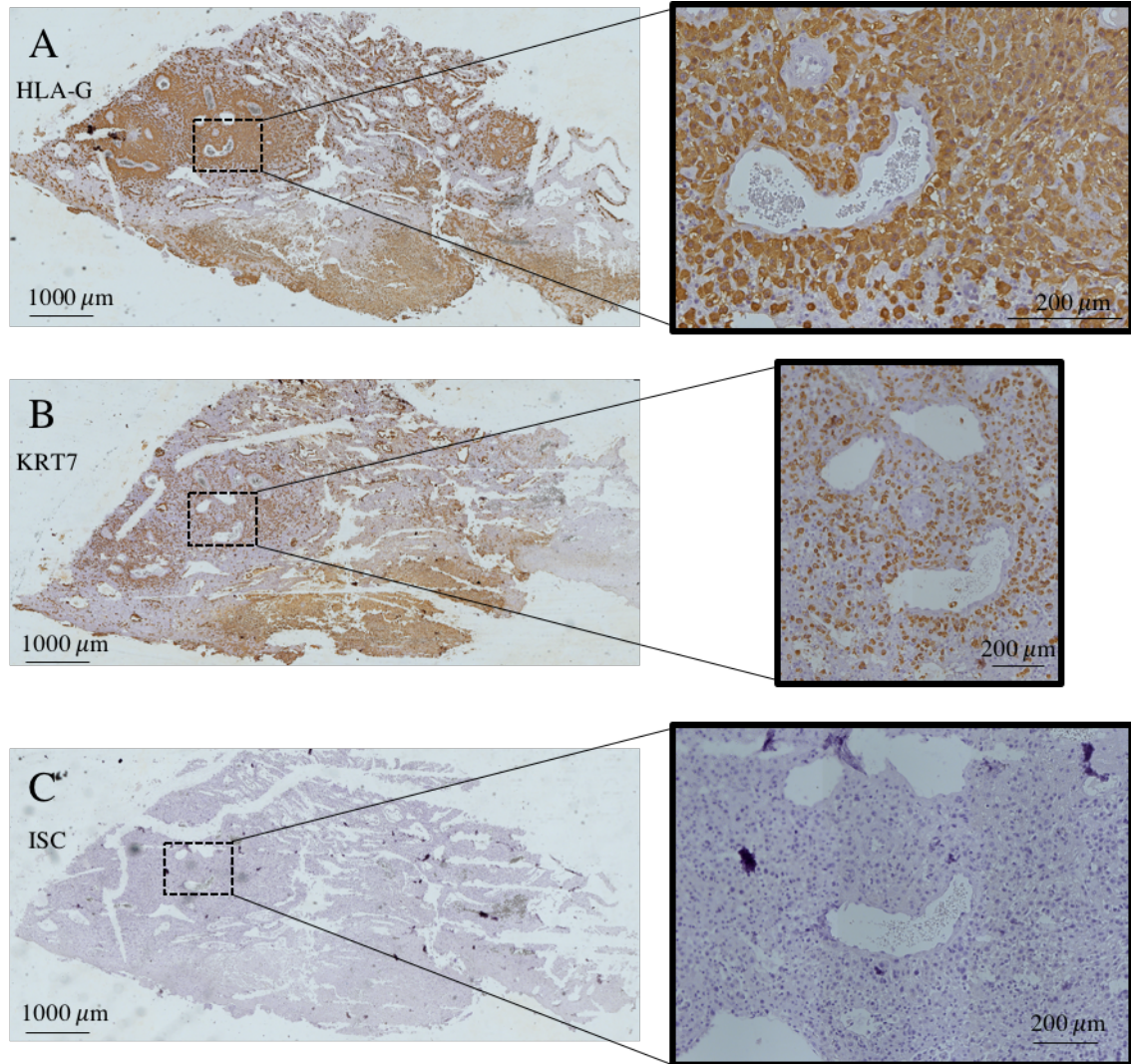


Figure 4.4: **Confirmation that tissue sampled was decidua basalis using immunohistochemistry.** (A) HLA-G is a marker for the extravillous trophoblast phenotype. (B) Cytokertain 7 is a marker for epithelial cells and stains positive in trophoblast cells and decidual glands. (C) Isotype control (ISC) with the same isotype and concentration as the primary monoclonal antibody, HLA-G. The higher magnification images show EVT cells surrounding arteries. Sample no: R008.

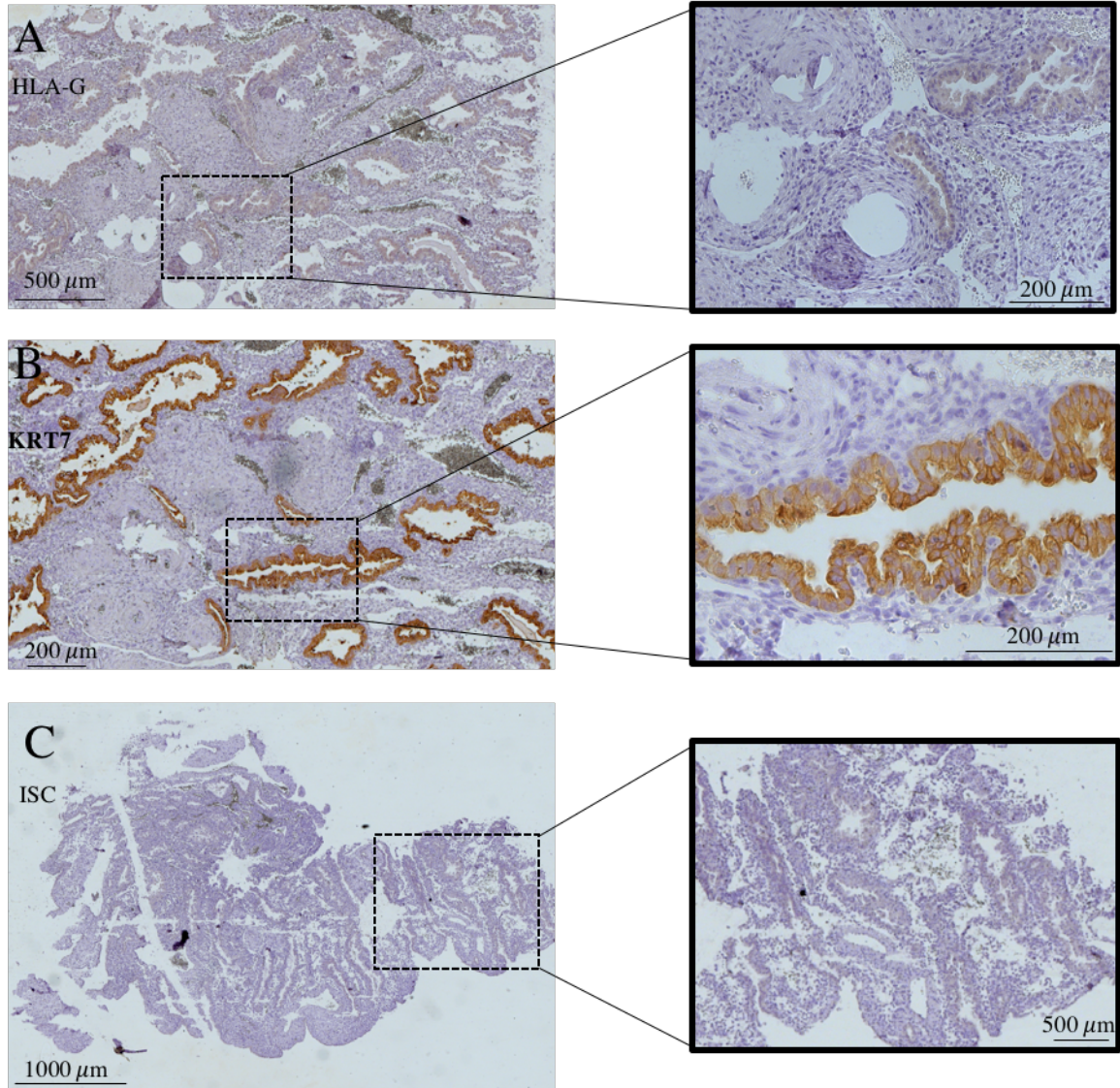


Figure 4.5: **Confirmation that tissue sampled was decidua parietalis using immunohistochemistry.** (A) HLA-G is a marker for the extravillous trophoblast phenotype and there is no positive staining. (B) Cytokertain 7 is a marker for epithelial cells and stains positive in trophoblast cells. Only epithelial cells at the periphery of the glands, stain positive. (C) Isotype control (ISC) with the same isotype and concentration as the primary monoclonal antibody, HLA-G. Sample no: R008.

4.4.1.2 Comparison of decidua parietalis and non-pregnant endometrium

Next, the stiffness of pregnant and non-pregnant endometrium was compared to ask if decidualization is driving a change in the tissue stiffness. For both these samples, a ‘soft’ cantilever was used during measurement (0.01 N/m). In humans, decidualization is uniform across the entire endometrium. This comparison is done with decidua parietalis, so it is independent from the implanting placenta. There was no difference in the apparent elastic modulus between these two tissues, with the medians measured at 199.4 Pa and 191.6 Pa for non-pregnant endometrium and decidua parietalis respectively (Mann-Whitney, N=5-6). Interestingly, this suggests it is the presence of the implanting placenta that is driving the increase in stiffness, measured in the decidua basalis. Individual data points for each non-pregnant endometrium sample is shown in Figure 4.6. Variation in apparent elastic modulus can be seen when comparing individual patient samples. This is most pertinent between sample E217, with a range of 10.77 and 1328.7 Pa and sample E219 with a range of 4.8 and 131.9 Pa. It is difficult to speculate whether this is due to true mechanical differences in these tissues or other factors such as location of the biopsy in the endometrium. Given these are patients with known fertility issues there may be pathology associated with the endometrium tested.

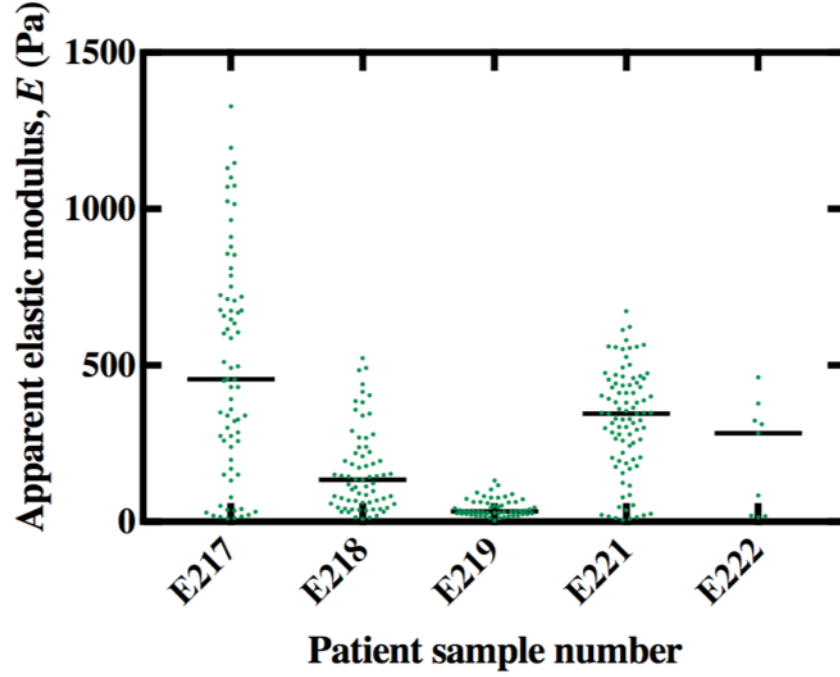


Figure 4.6: **Stiffness of non-pregnant endometrium.** Biopsies of non-pregnant endometrium were tested using the AFM to determine its apparent elastic modulus.

For each non-pregnant endometrium sample, the tissue was fixed and stained to confirm presence of luminal epithelium, the surface of the endometrium as described in materials and methods, chapter 3.4. A representative image from sample 221 is shown in Figure 4.7.

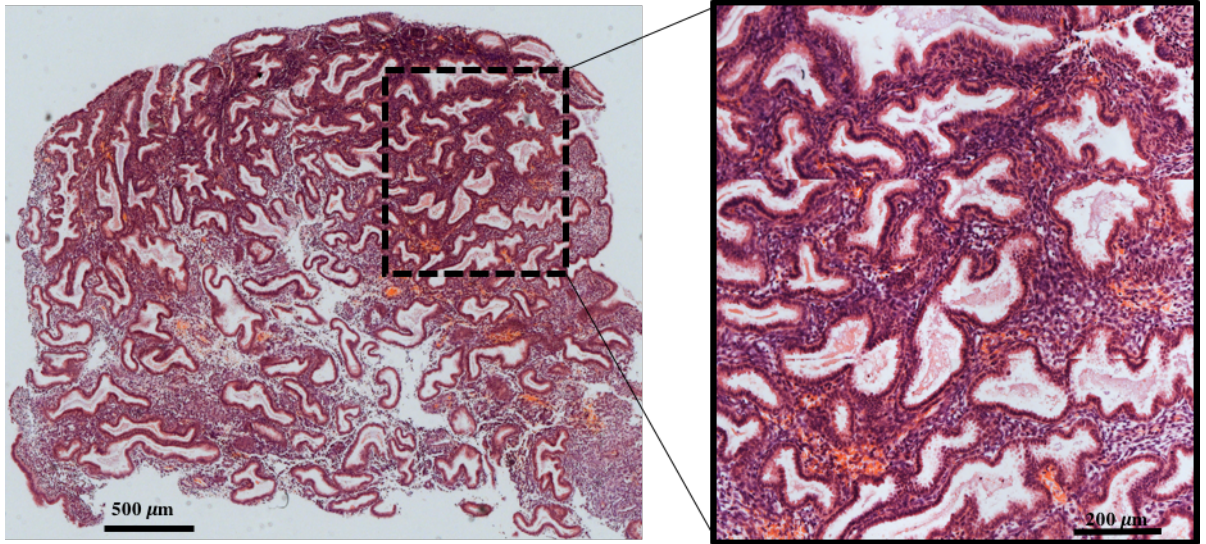


Figure 4.7: **Non-pregnant endometrium in the secretory phase of the menstrual cycle.** Hematoxylin and eosin stain shows the uterine glands in the endometrium. The glands appear zig-zag and folded, which is typical during the secretory phase. This compares to relatively straight and narrow glands during the proliferative phase.

4.4.1.3 Stiffness of first trimester placental villi

The median apparent elastic modulus of placenta villi isolated from 8-10 week pregnancies was found to be 246.5 Pa, which was in-between that of decidua basalis and decidua parietalis. A ‘soft’ cantilever (0.01 N/m) was used to measure the stiffness of placental villi. There were challenges in taking measurements of the villi because they are soft, thin strands, which tended to detach from the agarose gel it was embedded on upon contact with the AFM tip. This was overcome by partially embedding half the length of the villi in agarose. Individual data points for each non-pregnant endometrium sample is shown in Figure 4.8.

4.4.1.4 Summary of decidua, non-pregnant endometrium and placenta

Decidua basalis was found to be stiffer than decidua parietalis, non-pregnant endometrium and placental villi ($P < 0.0001$, Kruskal-Wallis ANOVA, Figure

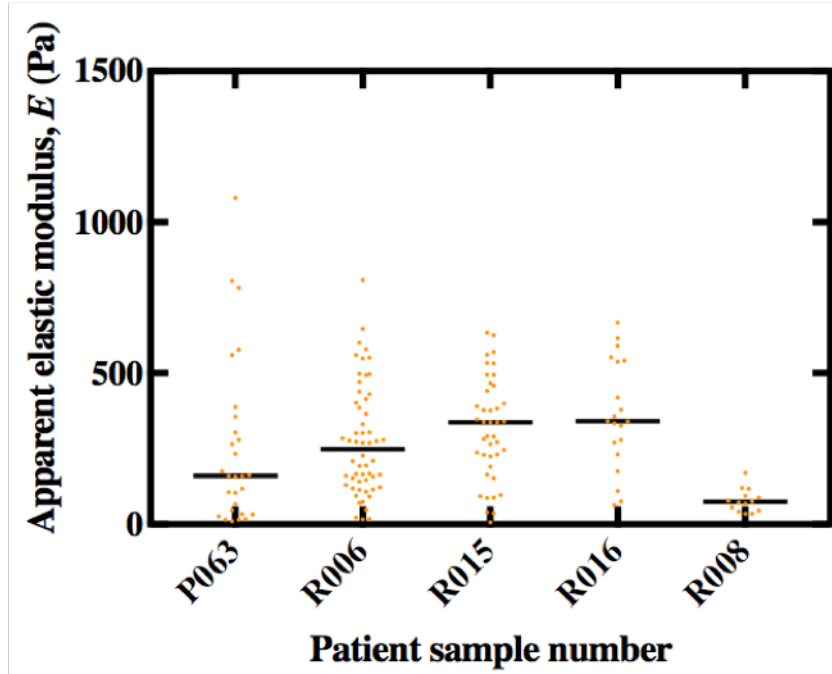


Figure 4.8: **Stiffness of first trimester placental villi.** Biopsies of placental villi were tested using the AFM to determine its apparent elastic modulus.

4.9). There was no difference in the stiffness measured between decidua parietalis, non-pregnant endometrium and first trimester placenta villi ($P > 0.05$, Kruskal-Wallis, multiple comparison test, Figure 4.9).

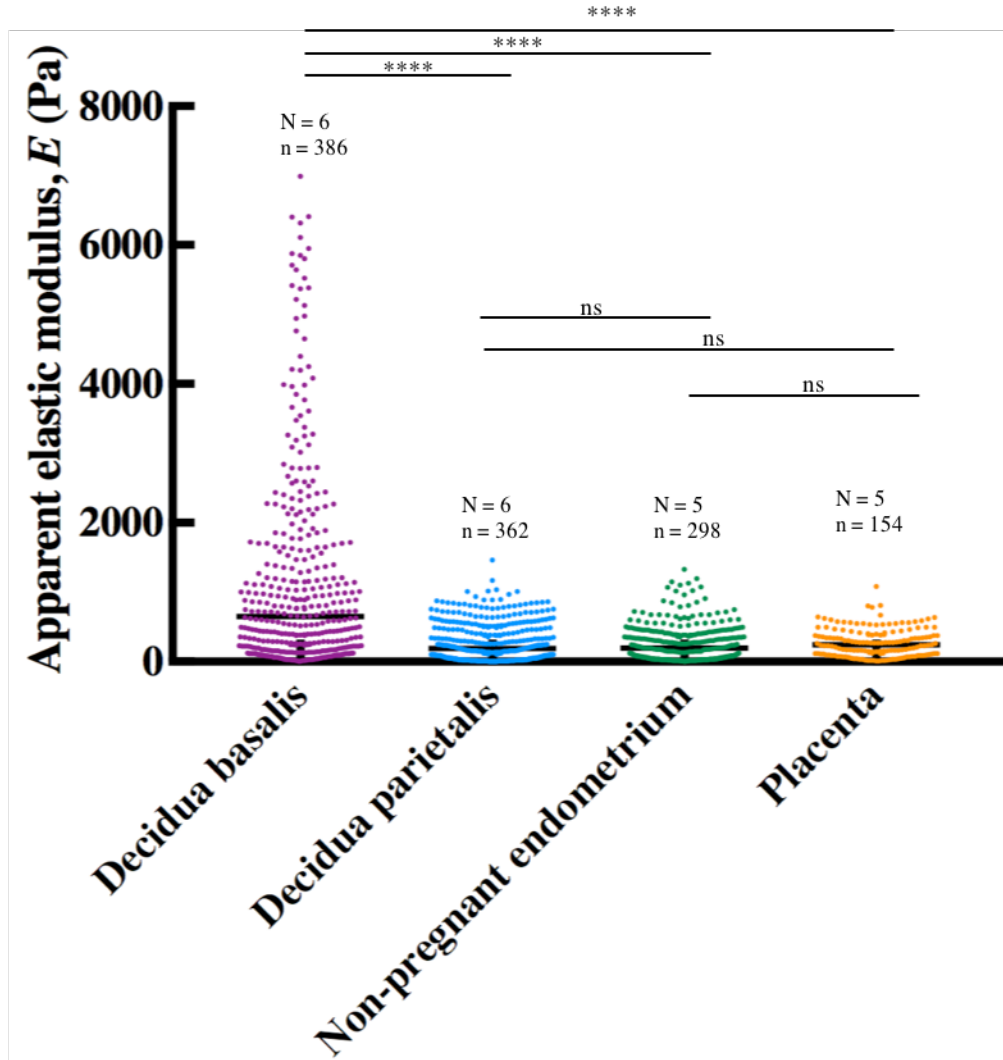


Figure 4.9: **Comparison of decidua, non-pregnant endometrium and placenta.** Decidua basalis was stiffer than decidua parietalis, non-pregnant endometrium and placenta villi. Median values, Kruskal-Wallis ANOVA with multiple comparison tests between groups, **** $P < 0.001$, ns = non-significant, N = number of patient samples, n = number of measurements.

4.4.2 EVT migration and stiffness

4.4.2.1 Stiffness of Matrigel

The stiffness of five Matrigel batches were determined using AFM (see materials and methods, chapter 3.5.1.2). A ‘soft’ cantilever (0.01 N/m) was used to measure the stiffness of Matrigel. For pure or 100% Matrigel the median apparent elastic modulus was found to be 202.9 Pa, which is 449 Pa lower than was found for the decidua basalis (Figure 4.10). Stiffness was determined for Matrigel diluted to 80%, 60% and 40% with EVT medium (HAMS-F12). A linear regression analysis was performed with $R^2 = 0.97$ and equation of the line $Y = 4.5x - 111.3$ (Figure 4.10). Different batches were tested because Matrigel is not a well defined matrix, which can produce a source of variability (Gjorevski et al., 2016). The range of apparent elastic modulus found for pure, 100% Matrigel was between 243.4 Pa and 512.4 Pa, a near doubling of the lower value.

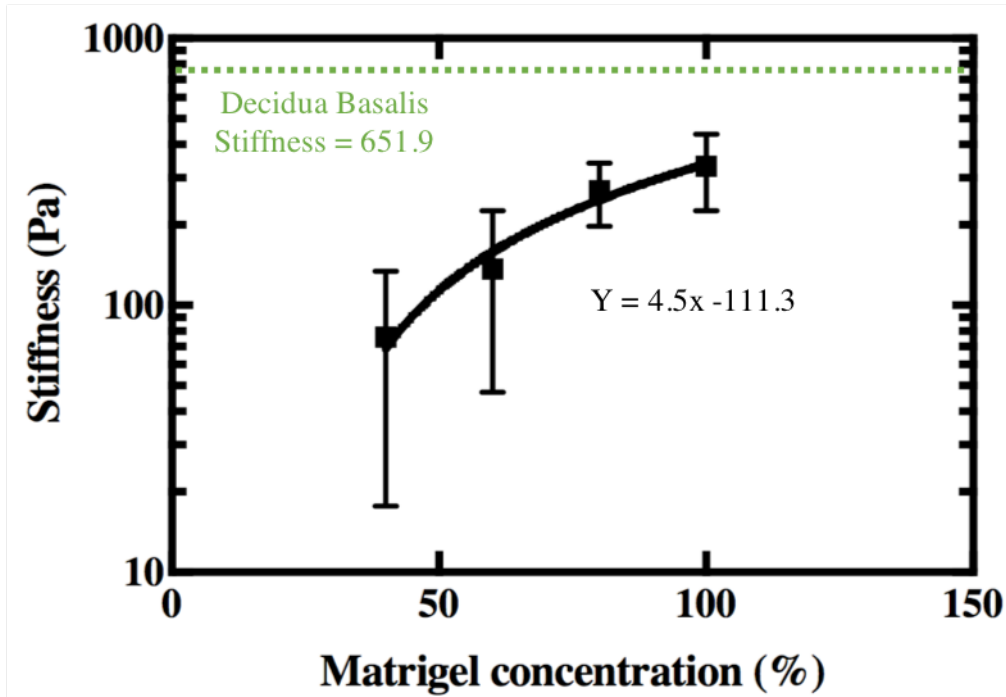


Figure 4.10: **Stiffness of Matrigel.** AFM was used to determine stiffness of Matrigel of five batches and at dilutions to 80%, 60% and 40% with EVT medium (HAMS-F12).

Figure 4.10 shows that Matrigel stiffness can be controlled by changing its concentration, but there are disadvantages to this approach. This will reduce ECM content, changing the density of ligand binding sites. In addition, concentration of growth factors contained in Matrigel would reduce. There was a clear need for an *in vitro* model in which stiffness alone can be altered, independent of ECM composition and Matrigel growth factor content.

4.4.2.2 Matrigel - alginate composite gel

The stiffness of Matrigel-alginate composites was determined using AFM (described in materials and methods, chapter 3.6.1.3) and the measurements were repeated from the protocol published by Chaudhuri *et al.*, for two reasons Chaudhuri *et al.* (2014). First, the alginate used by Chaudhuri *et al.*, was not available for use in this study and the mechanical properties are known to depend on the source of the alginate derived seaweed (Fu *et al.*, 2011). Second, because the stiffness of Matrigel was found to vary between batches (Figure 4.10) a single batch of Matrigel should be used for all experiments. For consistency, the same ‘soft’ cantilever (0.01 N/m) was used to determine stiffness for all Matrigel-alginate composites. An illustration of the interpenetrating network of Matrigel-alginate composite, with alginate cross-linking by the addition of Ca^{2+} ions, is shown in Figure 4.11A.

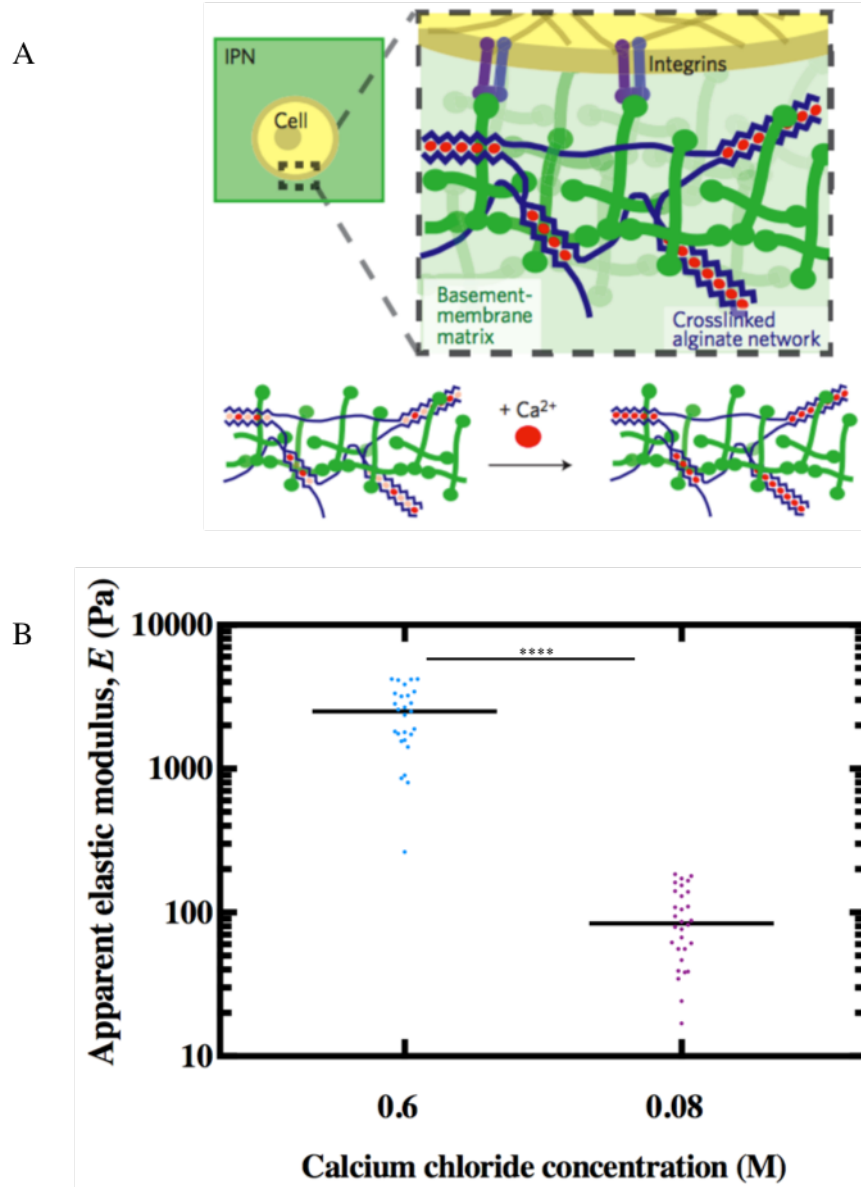


Figure 4.11: **Matrigel - alginate composite gel.** (A) An interpenetrating network of Matrigel and alginate was used to control matrix stiffness independent of ECM protein concentration. (B) Stiffness can be tuned with the concentration of Ca²⁺ ions in calcium chloride solution, used to crosslink the alginate network. Median, *** $P < 0.001$, determined using the paired t-test. Figure 4.11A was taken from (Chaudhuri et al., 2014).

Equal volumes of calcium chloride at concentrations between 0.02 M and 0.6 M

were added to a 1:1 (v/v) ratio of Matrigel - alginate. For simplicity, two calcium chloride concentrations were taken forward to be used with primary EVT, 0.6 M and 0.08 M with the median apparent elastic modulus found to be 2504.4 Pa and 84.1 Pa respectively (Figure 4.9B). The data was found to follow a gaussian distribution, determined using the D'Agostino & Pearson normality test. The stiffness at 0.08 M is at the same order of magnitude as decidua parietalis and 0.6 M was chosen as the stiff condition. Fabricating 3D substrates that are reliably 651.9 Pa and 191.6 Pa, matching the stiffness of decidua parietalis and basalis found in this chapter, is incredibly difficult, even with simple substrates such as polyacrylamide.

4.4.2.3 EVT migration on substrates of varying stiffness

EVT isolated from three primary samples at gestational age 8-9 weeks were encapsulated in the Matrigel - alginate composites and incubated for 1.5 h at 37 °C after gelling. The cells were then imaged every 20 min for 2.33 h in 3D (z-distance of 120 μm) using a confocal laser scanning microscope. Only HLAG+ cells, confirmed using immunofluorescence, were used to determine the velocity. There was an increase in the mean cell speed ($n=50-76$) for all three repeats, at the higher stiffness gel (Figure 4.10A). The mean speed at 0.6 M and 0.08 M was $8.9 \pm 4.9 \mu\text{m/h}$ and $7.6 \pm 5.5 \mu\text{m/h}$ respectively (Figure 4.12B). The speeds are at the same order of magnitude as EVT cells imaged for 12 h in a microfluidic device, when under a cytokine gradient ($6.26 \mu\text{m/h}$) and for no gradient ($6.52 \mu\text{m/h}$) (Abbas et al., 2017). The data was found not to follow a gaussian distribution using the D'Agostino & Pearson normality test, therefore non-parametric tests were used to assess statistical significance. No significance was found between substrates of varying stiffness over three patient samples (Mann-Whitney, $N = 3$, Figure 4.12D). The difference in mean cell speed for sample R048 was marginal, with an increase of $0.11 \mu\text{m/h}$.

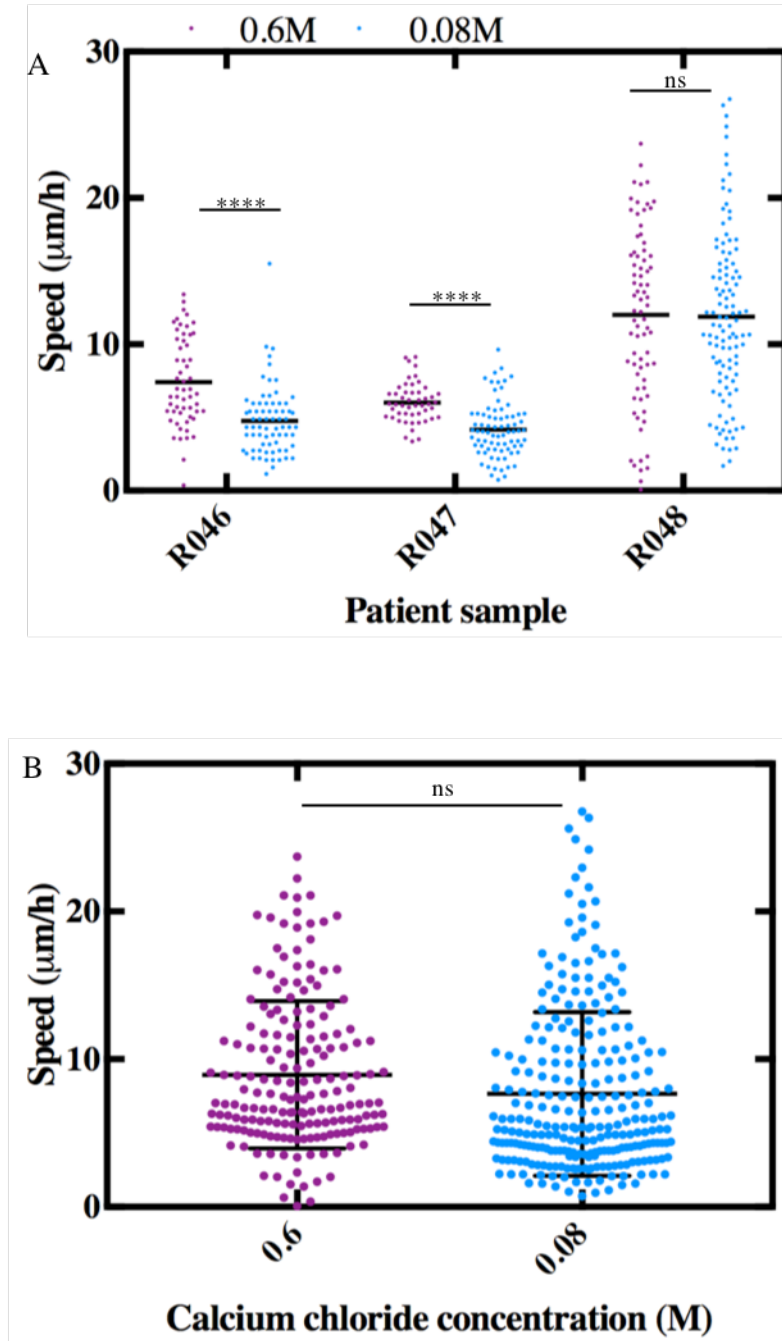


Figure 4.12: Speed of EVT cells migrating in Matrigel-alginate gels of different stiffness. A) The speed of HLAG+ EVT from three primary samples at gestational 8-9 weeks. Mean $\pm$ SD, **** $P < 0.0001$, ns = not significant, determined using the wilcoxon test $n = 50-76$ cells (B) No significant difference in cell speed of varying substrate stiffness. Mean $\pm$ SD, determined using the Mann-Whitney test, $N = 3$ tissue samples.

4.4.3 ECM architecture in the decidua

4.4.3.1 Whole mount staining of the decidua

Whole mount staining is the staining of small pieces of tissues without sectioning. This is a common technique used in embryo and zebrafish research. However, staining the decidua is more challenging as the tissue is bathed in maternal blood after the termination procedure. A piece of decidua basalis (1 cm x 1 cm) was first identified and a combination of resting the tissue in medium (30 min, RPMI) and rotating the tissue enclosed in an eppendorf tube, (30 min) resulted in no visible blood. The tissue was then fixed for 48 h, washed in sucrose buffer to stop fixation and stained before imaging (see materials and methods, chapter 3.4.4, for full protocol). The tissue was stained with a single antibody for collagens I, II, III, IV and V (in red), DAPI nuclei (in blue) and EPCAM (in green) for epithelial cells. Figure 4.11 and Figure 4.12 are 3D and 2D images respectively taken with a LSM confocal microscope, which are likely glands because of the size (in comparison to the glands in Figure 4.4B) and the positive EPCAM staining around its periphery.

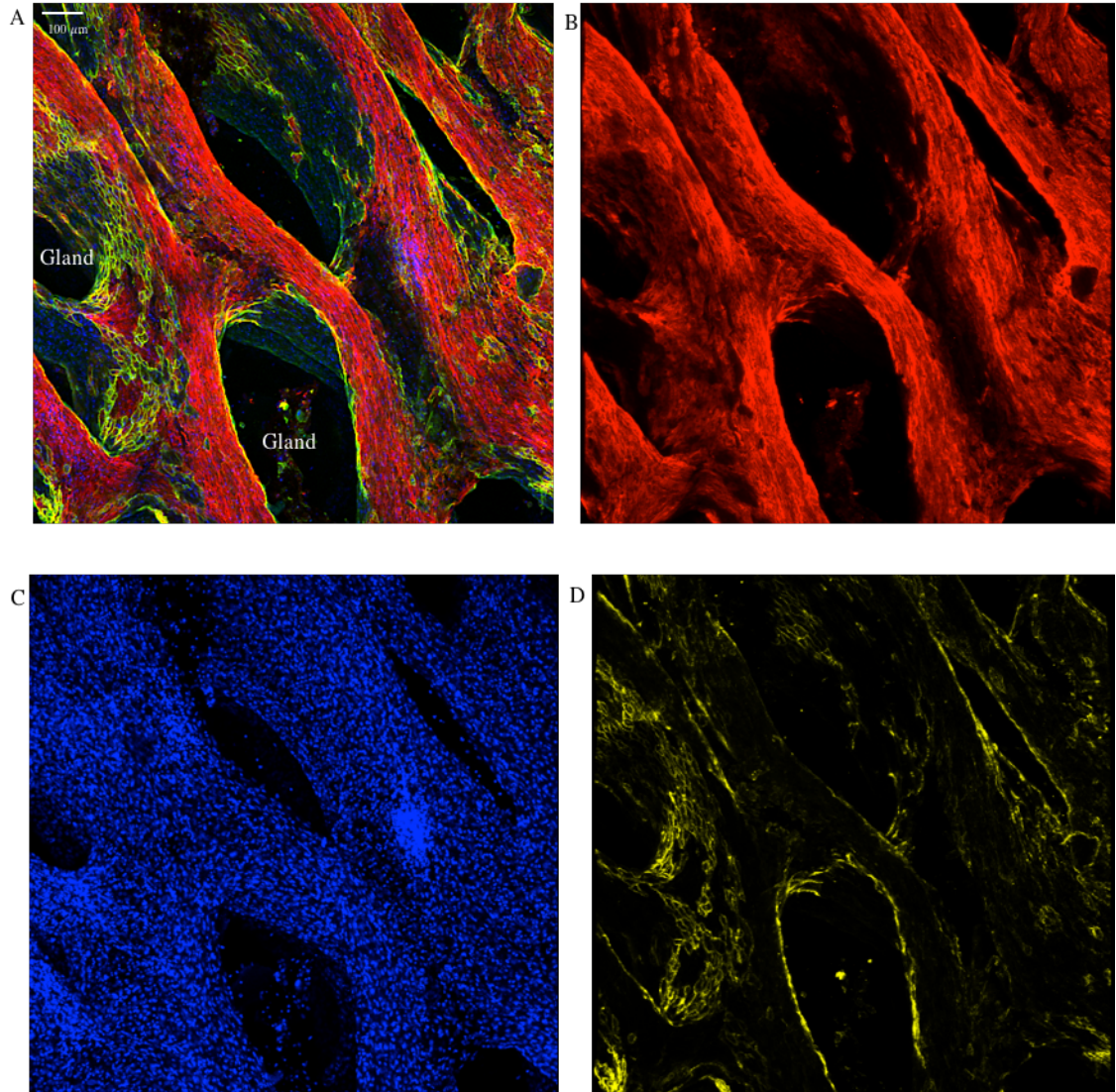


Figure 4.13: **Whole mount staining of human decidua in 3D.** A) Merged image of all immunofluorescence antibodies, which include (B) Collagens I, II, III, IV and V (C) DAPI nuclei and (D) EPCAM for epithelial cells. Images are in 3D with a z-distance of 121 μm .

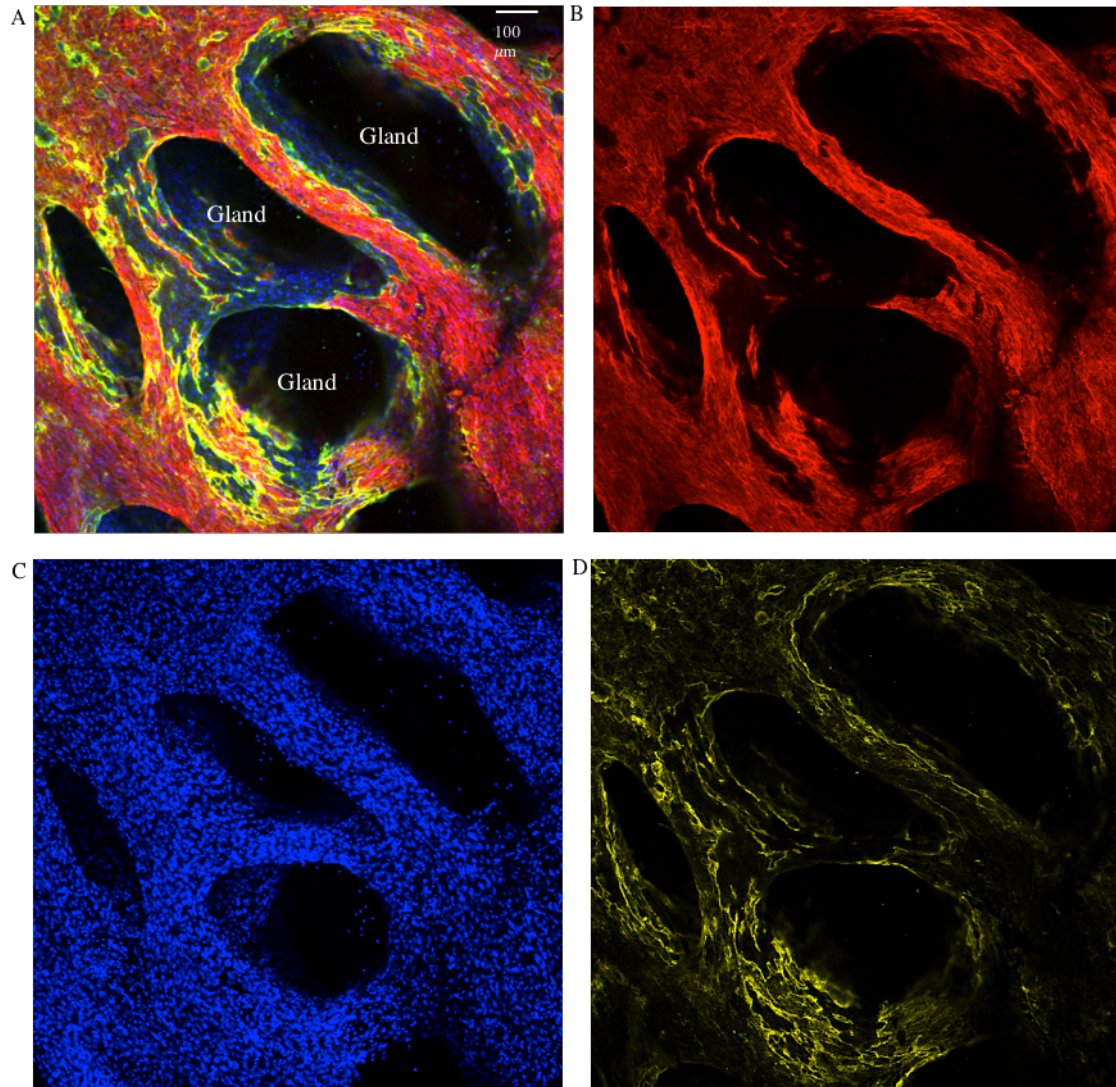


Figure 4.14: **Whole mount staining of human decidua in 2D.** A) Merged image of all immunofluorescence antibodies, which include (B) Collagens I, II, III, IV and V (C) DAPI nuclei and (D) EPCAM for epithelial cells. Images are in 2D.

4.5 Discussion

4.5.1 Stiffness at the maternal-fetal interface

Decidualization in humans is characterised by dramatic tissue remodelling and is regulated by hormones secreted during the menstrual cycle in preparation for pregnancy (Benirschke *et al.*, 2012; Sharma *et al.*, 2016). In this chapter, decidua basalis was found to be stiffer compared to decidua parietalis in all six patient matched samples. When the median of all data points are taken, this represents a 3-4 fold increase. In contrast, the difference between decidua parietalis and non-pregnant endometrium was 7.8 Pa. This suggests that there is no dramatic change in stiffness during decidualization and the increase in stiffness of decidua basalis is a consequence of the implanting placenta. It is important to note that non-pregnant endometrium samples were obtained from biopsies of women with infertility issues. Therefore, mechanical changes could result as a consequence of pathology. Samples from healthy women are difficult to obtain but this would be necessary, to say for certainty, what the effect decidualization has on the mechanics of the endometrium.

There are a number of explanations that could explain the increase in tissue stiffness between decidua basalis and parietalis. First, decidua basalis is characterised with the presence of invading HLA-G+ cells, which produce ECM proteins. For example, Oefner *et al.*, found collagen IV is significantly up-regulated in decidua basalis versus parietalis, compared to collagen I, III and VI, and invading EVT cells in the decidua uniquely secrete collagen IV (Oefner *et al.*, 2015). Second, Talaulikar *et al.*, found collagen fibrils were significantly thicker in the stroma of decidua basalis compared to those in the decidua parietalis (Sinai Talaulikar *et al.*, 2014). Interestingly, these differences were observed between 9 and 12 weeks gestation but not at gestations below 9 weeks. The authors speculate that this is a result of decidual control or limiting of trophoblast invasion, or as a form of physical support for the developing feto-placental unit. The gestational age of decidual samples in this study were at 9 weeks $\pm$ 5 days, therefore no comparison can be made with the study by Talaulikar *et al.* Both the presence of additional cells and the deposition of collagen IV would result in an increase in tis-

sue stiffness. However, Collagen IV around EVT is globular, which is markedly different to the classic distribution of collagen IV, which is the primary ECM protein in basement membrane and forms a network of reticular fibrils (Li and Thompson, 2003). Moreover, collagen IV is not known for providing mechanical strength in tissues, therefore it would be surprising if this alone contributes to the increased stiffness found in this chapter. A similar globular appearance of this collagen type has previously been reported around certain cancer cells, and trophoblast and malignant cells are known to share the characteristics of invasion (Kondo et al., 2012; Zhu et al., 2012).

An alternative explanation for the increase in stiffness is changes in the vasculature between decidua basalis and parietalis resulting from remodelling by EVT. For example, there is an increase in arterial medial thickness and medial necrosis and replacement with fibrinoid material Kam et al. (1999). However, the latter only occurs in the presense of interstitial trophoblast cells and the mechanics of fibrinoid in tissue explants remains uncharacterised. Therefore, it is difficult to speculate on the contributing effect of fibrinoid deposition on tissue stiffness. Further experiments are required to better understand what is contributing to the increase in stiffness in decidua basalis. The AFM can be coupled with a fluorescent light microscope and antibodies added to localise measurements. For the decidua, it would be useful to stain the tissue with HLA-G, cytokeratin 7 and endothelial cells to identify EVT, glands and vessels. For HLA-G, the G223 antibody can be used without fixation as demonstrated in chapter 5.4.1. However, significant challenges need to be overcome to make this possible. The decidua is initially very bloody and not well suited to microscopy in comparison to neural tissue (Koser et al., 2016). Longer incubation steps with agitation as demonstrated in the whole mount staining in chapter 4.4.3.1 would be required or chemicals to help with calcification. However, there are concerns with matrix degradation with both of these methods.

4.5.2 Stiffness and trophoblast migration

The results presented in this chapter show that there was no difference in trophoblast migration in 3D substrates of varying stiffness. Although, there was

a trend showing increasing stiffness promotes trophoblast migration, this trend was not found to be statistically significant. With only three samples, this is not surprising and therefore more repeats are necessary. Further experiments are required to determine whether stiffness is a regulator of trophoblast invasion. This experiment should be repeated on 2D on substrates of different stiffness albeit recognising the limitation of migration experiments on 2D. Secondly, on substrates with a stiffness gradient to ask whether EVT cells move towards or away from areas of increasing stiffness (Hadden et al., 2017). Lastly, migration of EVT along a chemokine gradient such as GM-CSF has previously been shown to direct EVT migration in 3D (Abbas et al., 2017). This experiment using microfluidics can be repeated but on substrates with varying stiffness to determine whether mechanics alters migration dynamics in the presence of a chemokine gradient.

The method using interpenetrating networks of Matrigel - alginate overcomes some of the limitations of previous studies: it is in 3D and stiffness is controlled independent of ECM protein concentration. The latter is important because the number of cell-matrix adhesions per cell is kept constant. The alginate networks restricts deformation of Matrigel due to tensional or protrusive forces by cells, thereby regulating local ECM stiffness around the cells. The mechanism by which cells sense local mechanics and translate it into intracellular responses is not fully understood (Wang and Chen, 2013). Studies with cells migrating on substrates with increasing stiffness have suggested that cells are able to probe the rigidity of the substrates, and subsequently activate stress-sensitive ion channels or a change in protein expression (Lo et al., 2000). As a consequence, this may alter the strength of contractile forces and thus migration. Increasing stiffness is thought to result in a slower migration speed as a result of a larger cell spreading area causing an increase in the number of cell-matrix adhesions per cell (Isenberg et al., 2009; Lange and Fabry, 2013). However, most of the evidence is based on cell migration on 2D substrates, which may explain the opposite trend in this study, which is in 3D. One hypothesis is substrates of higher stiffness result in an increased production of matrix metalloproteinases (MMPs), which breakdown ECM protein. The substrates used in this study are rich in ECM proteins, which is not the case for 2D studies done on synthetic substrates or a single layer of

ECM protein. Therefore the pathways that lead to MMP production may not be sufficiently activated in these 2D studies.

4.5.3 Substrates to study trophoblast cells

There is a need to move away from Matrigel towards synthetic substitutes with properties that can be tailored to different tissues and organs. As previously discussed, Matrigel is a ‘black box’ with variations in protein and growth factor content between batches. In this chapter, the stiffness of Matrigel was shown to vary between batches, which is in line with other literature (Soofi *et al.*, 2009). Recently, Gjoverski *et al.*, published a protocol detailing the fabrication of synthetic gels made from polyethylene glycol (PEG), where both its ECM content and stiffness can be finely tuned (Gjorevski *et al.*, 2016). In the context of trophoblast research, the ECM content of both first trimester placenta and decidua are already established. With the stiffness results from this study, a synthetic approach can now be used to tailor gels to study both trophoblast stem cell niche and EVT migration in well defined, physiological matrices.

4.5.4 Whole mount staining of the decidua

In this chapter, whole mount staining of the decidua has been demonstrated without the need for complex and long decellularization experiments. ECM proteins can be stained to view its architecture in 3D, which is not possible with immunohistochemistry sections and cells can be stained to localise the ECM. The resolution of the decidua could be improved with the use of a two-photon excitation microscope enabling increased penetration depth resulting from multi-photon absorption. This technique could be used to gain a better understanding of the distribution of collagen IV in 3D.

4.6 Conclusion

Tissue stiffness of non-pregnant endometrium, pregnant endometrium (decidua) and placental villi has been determined. The finding that stiffness in the decidua basalis increases as a result of the implanting placenta is further evidence of the

4.6 Conclusion

dramatic change that occurs during pregnancy. Further experiments are required to determine the factors that are contributing to this change. No difference was found in trophoblast migration in 3D substrates of varying stiffness. The mechanisms that convert mechanical signals into cell action remain poorly understood. More generally, these results provide direct measurements of the tissue stiffness at the maternal-fetal interface during the first trimester of pregnancy. This will allow bioengineers and biologists to design better experiments related to embryo implantation and trophoblast migration.

Chapter 5

Effects of low oxygen on trophoblast motility

The evidence on the effect of low oxygen on trophoblast motility is conflicting. This is in part because of the differences in methods used and the over-reliance on trophoblast cell lines, which are poor models of primary extravillous trophoblast cells. In this chapter, the effect of low oxygen on primary EVT cell motility is examined. The cells migrate in a three-dimensional (3D) matrix and only cells that have the HLA-G antigen, a marker for the extravillous phenotype, are analysed.

5.1 Introduction

Early fetal and placental development occurs in a low oxygen environment. In the first trimester of pregnancy, an oxygen gradient exists between the intervillous space (20 mmHg or 2.5%) and the maternal decidua (60 mmHg or 8.4%) with the oxygen tension at the intervillous space rising to 60 mmHg by 12 weeks, as a result of the onset of maternal arterial circulation (Jauniaux *et al.*, 2000). Before this onset, cell metabolism is low and the feto-placental unit is supported by secretions from the endometrial glands (Burton, 2009). Under the low oxygen environment, cytotrophoblast proliferation and differentiation into the invasive pathway takes place. Extravillous trophoblast (EVT) cells migrate from the tip of the anchoring villus and invade the decidua. There has been extensive interest in oxygen as a regulator of trophoblast invasion but the evidence is conflicting with large differences in experimental procedures (Caniggia and Winter, 2002; Genbacev *et al.*, 1996; Fitzpatrick and Graham, 1998; Kadyrov *et al.*, 2003; Graham *et al.*, 2000). The central question of all these *in vitro* studies is: does low oxygen stimulate or inhibit trophoblast invasion? In this chapter, EVT motility under different oxygen concentrations is investigated with an attempt to address the limitations of previous studies.

5.1.1 Low oxygen and trophoblast invasion

Oxygen-mediated regulation of trophoblast invasion is complex and its pathway is not yet clear. Most studies conclude low oxygen ($\leq 3\%$) inhibits invasion, determined by the outgrowth of trophoblast cells from explant cultures. Caniggia *et al.*, found this is mediated through up-regulation of Hypoxia-Inducible Factor (HIF), which up-regulates transforming growth factor beta-3 (TGF β 3) expression, thereby inhibiting trophoblast differentiation into invasive EVT (Caniggia *et al.*, 2000). This is consistent with Genbacev *et al.*, who found cytotrophoblasts failed to differentiate into invasive EVT, in part due to their inability to completely switch their repertoire of integrin cell Extracellular matrix (ECM) adhesion receptors (Genbacev *et al.*, 1996).

Contradictory findings were found by Fitzpatrick *et al.*, and Graham *et al.*, who found low oxygen mediates invasion linked to increased activity of plasminogen activator 1 (PAI)-1 and uPA system respectively (Fitzpatrick and Graham, 1998; Graham *et al.*, 2000). This is in line with Kadyrov *et al.*, who observed increased invasion depth of EVT in women with severe anaemia at term compared to pre-eclamptic and normal pregnancy (Kadyrov *et al.*, 2003). These histological observations suggest a perturbed trophoblast biology in anaemic pregnancies but the authors assume maternal anemia is correlated to lower oxygen levels at the maternal-fetal interface and make no direct measurements.

Differences in experimental conditions could explain the inconsistency. Low oxygen concentrations used in culture range from 0.1% to 3% with a control of 21%, where HIF-1 signaling, a transcription factor that drives hypoxic gene expression is at its maximum activity between 0.5-2% (Jiang *et al.*, 1996). The type of incubator, position of culture plate, volume of cell medium and time allowed for oxygen levels to equilibrate are all known to affect the concentration of oxygen cells experience. Direct comparison is further complicated by the addition of fetal bovine serum by Fitzpatrick *et al.*, and not others, which is known to influence cell differentiation and proliferation and is one of the regulators of HIF production (Pringle *et al.*, 2009). Many culture systems are serum free because the composition of serum varies from batch to batch. However, the urokinase-type plasminogen activator (uPA) or uPA system, responsible for the production of enzymes that degrade extracellular matrix cannot operate in the absence of plasminogen in serum. Therefore, the presence or absence of serum will affect which pathways are active or not. There are questions over comparing the response of trophoblast cell lines to explant cultures, given they have been shown to differ substantially at the transcription level from EVT (Apps *et al.*, 2011). There are contradictions on whether the JAR cell line responds to oxygen at all (Lash *et al.*, 2007; Zhang *et al.*, 2012). More recently, HTR/SVneo cells have been shown to be a heterogeneous population of trophoblast and non-trophoblast cells (Abou-Kheir *et al.*, 2017). Therefore, it is not clear which population is selected for or affected by low oxygen.

5.2 Model requirements to study EVT motility under low oxygen

5.2 Model requirements to study EVT motility under low oxygen

To avoid these confounding variables, experiments designed to investigate EVT motility under low oxygen should incorporate the following features. Primary cells from first trimester pregnancies should be used for studying EVT motility under low oxygen, given the doubts over relevance of trophoblast cell lines. Cell motility should be quantified either by measuring cell outgrowth from explant cultures or EVT isolated from placental tissue. Isolated EVT is preferred, as outgrowth is a poor measure of cell motility. Results are mostly qualitative with most studies commenting on whether outgrowth has increased or decreased under low oxygen. Quantifying outgrowth is challenging as it depends on the size of the placental villi tested and number of viable cells on the periphery of the villi. For isolated EVT, the cells should be stained to confirm the presence of HLA-G antigen, a HLA class I molecule uniquely expressed by invasive EVTs, given the heterogeneity of the primary cell isolate. The cells quantified should be single cells and not in clumps, as this affects migration dynamics (Friedl and Gilmour, 2009). The experiment, should be conducted in 3D for physiological relevance and with ECM proteins that are close to the *in vivo* microenvironment. It is important to note that although only HLAG+ cells will be tracked, the primary isolate contains a heterogeneous cell population made up of mesenchymal cells and lymphocytes. These cells will produce factors such as cytokines that may influence trophoblast migration.

The experiment should be long enough to allow the medium to equilibrate to the environmental oxygen concentration and for cells to sense, change gene expression and adapt to low oxygen. Newby *et al.* reported an equilibrium time of 4 hours (h) when transferring 125 ml of medium from 18% to 6-8% oxygen and 24 h to reach 2% (Newby *et al.*, 2005). When cells are cultured long term (5-7 days) and require medium change every 2-3 days the cells may experience ischemia-reperfusion injury with reoxygenation and it will take time for both the incubator and medium to equilibrate back to low oxygen conditions. Therefore, a low volume is required for rapid equilibrium of oxygen or pre-equilibration of

medium. For biological relevance, the experiment should be long enough for hypoxia to induce a change in gene expression and for gene expression to translate to physiological behavioural change. Chi *et al.*, carried out a time-dependent study on the genomic response of five cell types to hypoxia and found they have an approximately 2-3 fold change in mRNA expression after 6 h (Chi *et al.*, 2006). Therefore, an experiment time of at least 6 h is required for biological relevance, but a time-dependent study is required for primary EVT, as this will vary between cells. This study should investigate both gene expression changes and protein production. Finally, to prevent ischemia-reperfusion injury, the oxygen concentration in the incubator should be reduced gradually and an experiment should be no longer than 48 h to avoid medium change.

5.2.1 Single cell migration

In this study, single cell migration is investigated with the aim to model EVT cells that have escaped the distal column and are migrating as single cells. In practice, it is easier to track single cells than cells that migrate collectively or in clumps. However, it is important to recognise that cells migrating collectively in different shapes and sizes or as single cells will exhibit distinct migratory behaviours (George *et al.*, 2017; Barriga *et al.*, 2018). For example, Wong *et al.*, demonstrated that cells that have undergone epithelial-mesenchymal transition (EMT) migrate through engineered micropillars differently (Wong *et al.*, 2014). Individual cells with few neighbours exhibit faster and straighter trajectories and disperse away from the cells migrating collectively. In addition, the authors found a sub-population of collectively migrating cells that overexpressed epithelial biomarkers. Finally, it is important to note that dissociation of tissue to isolate single cells may itself drive gene expression changes (van den Brink *et al.*, 2017).

5.3 Design of model

5.3.1 Oxygen saturation

To study EVT motility under low oxygen, incubation at 1% oxygen saturation was chosen. This is lower than physiologically measured at 2.5% at the intervill-

5.3 Design of model

lous space, but concentrations of between 1-3% are considered appropriate when studying the effects of low oxygen on trophoblast. At the lower limit, it is hypothesised that up-regulation of genes associated with hypoxia would be greater, thus any downstream effects on cell migration would be more clear. Oxygen concentration of 8% was chosen as the control group, as atmospheric conditions are considered hyperoxic and not physiological to the placenta. In addition, this equates to the oxygen tension within the normal placental bed (Jauniaux et al., 2000). However, EVT motility was also measured at 21%, as it is useful to compare migration dynamics in this experiment, with previous time-lapse experiments, which were conducted at atmospheric oxygen.

An experiment length of 24 h was chosen, which allows time for medium to equilibrate and cells to sense, react and respond to a change in oxygen tension. At the same time, no medium change is required, which will avoid ischemia-reperfusion injury. For the 1% condition, cells are placed at 15% and the oxygen concentration is gradually reduced every hour to 10, 5 and finally 1% in order to reduce stress to cells. For the 8% condition, cells were placed straight into an 8% incubator, as this oxygen concentration is not considered hypoxic to EVT.

5.3.2 Culture dish

An IBIDI 4-well culture dish was chosen to house the cells for this experiment (Figure 5.1). The coverslip at the bottom of the insert is polymer based with a thickness of 180 μm , which makes for better optical clarity compared to well plates. There are 4 wells per dish, which allows for experiments to be conducted in parallel. Each well has a volume of 10 μl and the medium that sits on top of the well has a volume of 200 μl . The low volumes preserve the number of primary cells and promotes fast equilibrium of medium to bulk oxygen in the incubator. The depth of each well is 1.5 mm allowing for 3D cell migration and imaging in cell growth hydrogel (Matrigel, Corning, NY, USA). This hydrogel, a basement membrane matrix was chosen as it contains collagen-IV, a protein shown to be present in the decidua and known to influence the invasive behaviour of trophoblast cells (Oefner et al., 2015). The 4 wells sit in a dish with an outer

volume of 1 ml, which creates a humid environment and prevents dehydration of the gel.

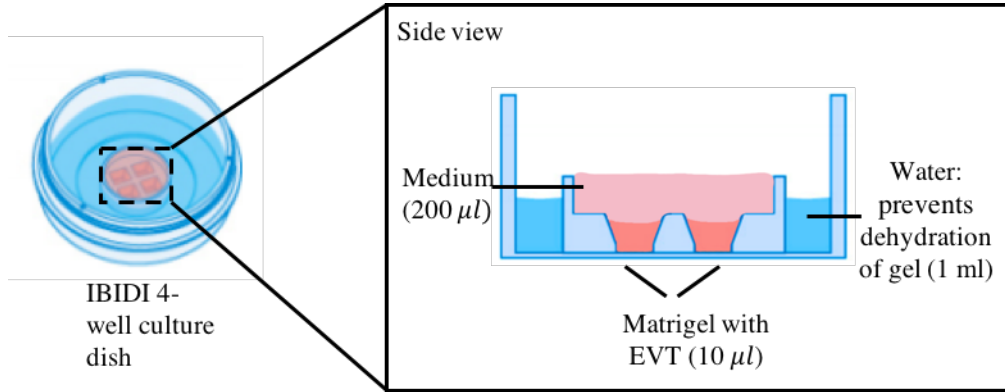


Figure 5.1: **Culture dish.** The cell isolate is encapsulated in Matrigel and pipetted into an IBIDI 4-well culture dish, with a volume of 10 μ l per well. 200 μ l of medium sits on top of the 4 wells and 1 ml of water around, to prevent dehydration. The culture dish is sealed and placed in 37 °C incubators at 21, 8 and 1% oxygen saturation. Image adapted from IBIDI.com

5.3.3 Cell tracking

5.3.3.1 Challenges

Cell motility was tracked using a laser scanning confocal microscope, which enables the reconstruction of 3D structures from images taken at different z-positions. There were two routes to tracking cell motility under low oxygen: (1) placing a confocal microscope inside a low oxygen chamber or (2) generating low oxygen within a microscope insert. Light microscopes can be placed within low oxygen chambers, but confocal microscopes are bulkier due to the addition of lasers. Moreover, this route was not practical as it required two chambers and two microscopes, if the control and test condition were to be done in parallel.

For the second route, a closed insert needed to be designed to maintain the desired oxygen concentration. Otherwise an oxygen controller is needed to continually sense and adjust oxygen levels. However, no such microscope insert exists commercially. The challenge would be to fit it around a culture plate and for it

not to interfere with the microscope's objective. In chapter 6, a system to produce an oxygen gradient within a microfluidic device, which can be placed under a microscope was designed. Here, no bulky gas bottles are required, as an oxygen gradient was delivered by deoxygenated liquid. However, it would be challenging to maintain an oxygen concentration level of between 1-3% in a large enough volume, due to the high permeability of materials used to manufacture microfluidic devices. Therefore, a solution whereby cell motility is quantified when cells are placed in a regulated oxygen chamber over a 24 h period is required.

5.3.3.2 Mean square displacement

In the proposed experiment, the mean squared displacement (MSD) of a cell is calculated. MSD is the mean distance a cell travels from its starting point. It is most commonly used in particle physics to determine whether particle movement obeys random walk or if it is directed. The MSD of a cell in three dimensions is given by:

$$MSD = [\Delta \vec{r}(t)^2] = \frac{1}{N} \sum_{i=0}^N (\vec{r}_i(t_0 + \Delta t) - \vec{r}_i(t_0))^2 \quad (5.1)$$

where $\vec{r}_i = (x_i, y_i, z_i)$ denotes the cartesian coordinates of the i th cell, t_0 denotes the start time, and $(x_i(t_0), y_i(t_0), z_i(t_0))$ corresponds to the initial position of the cell (Li et al., 2010). The MSD is averaged over all cells at time $t = t_0 + \Delta t$ and $[\Delta \vec{r}(t)^2]$ denotes the arithmetic mean of the square distances. The MSD is illustrated in Figure 5.2A for particle diffusion in 1D and in Figure 5.2B for cell movement in 3D. For this model, cells were imaged to obtain their coordinates at $t_0 = 0h$, before they are placed at 1, 8 and 21% oxygen concentration. They were left for 24 h and imaged once more to obtain coordinates at $t = 0h + 24h$. For easier interpretation, the square root was taken of the MSD and referred to as the root mean square displacement (RMSD). The hypothesis is that there is a statistical difference in the RMSD between the control (8% oxygen) condition and low oxygen (1% oxygen) condition.

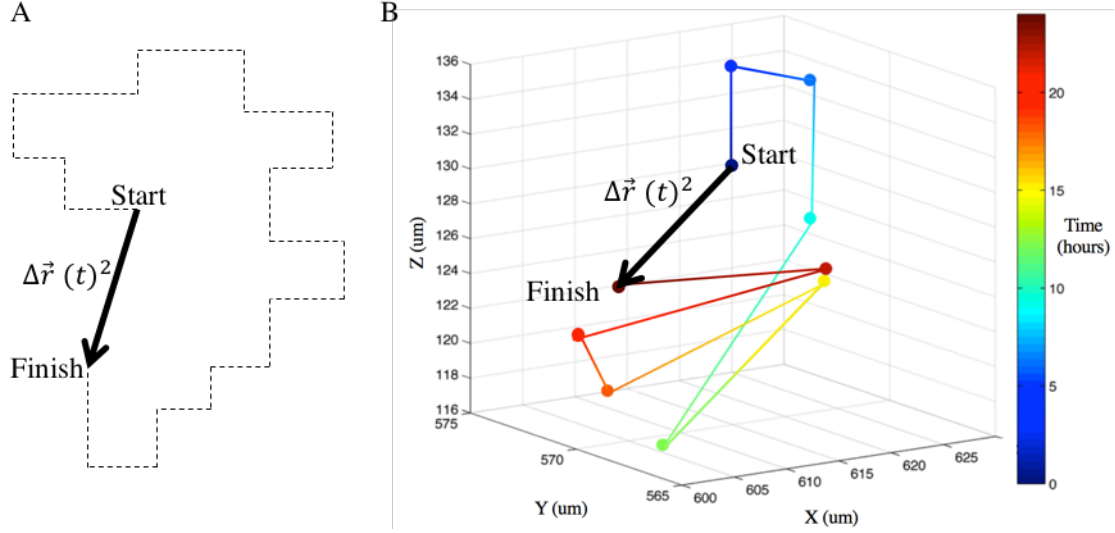


Figure 5.2: **Mean Square Displacement.** MSD is the mean distance a particle or cell travels from the starting point and is illustrated in a (A) 2D plot and (B) 3D over a 24 h time period.

5.3.3.3 Point set registration

After incubation, the challenge was to image the same volume of gel and the same HLAG+ cells (Figure 5.3A). The wells were aligned so that the x, y plane of view was the same at 24 h as at 0 h. However, it was not possible to position the culture plate in the same area, even with alignment markers. The smallest deviation results in bias when quantifying cell motility. To overcome this, point set registration, the process of finding spatial transformation that aligns two point sets was used. In this assay, the two point sets are fluorescent beads, which are inert and immobile. Figure 5.3B is an illustration of point set registration, where (i) is a 2D image, (ii) is the same image rotated, (iii) is an overlay of the outline of both images and (IV) is the result after point set registration.

For this study, coherent point drift (CPD), a point set registration algorithm developed by Myronenko and Song was used (Myronenko and Song, 2009; Wolfahrt, 2017). The principle is based on the maximum likelihood estimation, a statistical model that finds parameter values that best explain the observed data. Python programming language was used to identify the fluorescent beads (FB) at

5.3 Design of model

0 h and 24 h and CPD analysis was run. The transformation is described mathematically and applied to the cell coordinates at 24 h. Because the fluorescent beads are immobile, any deviation of the cells from the original coordinates is due to cell movement.

5.3 Design of model

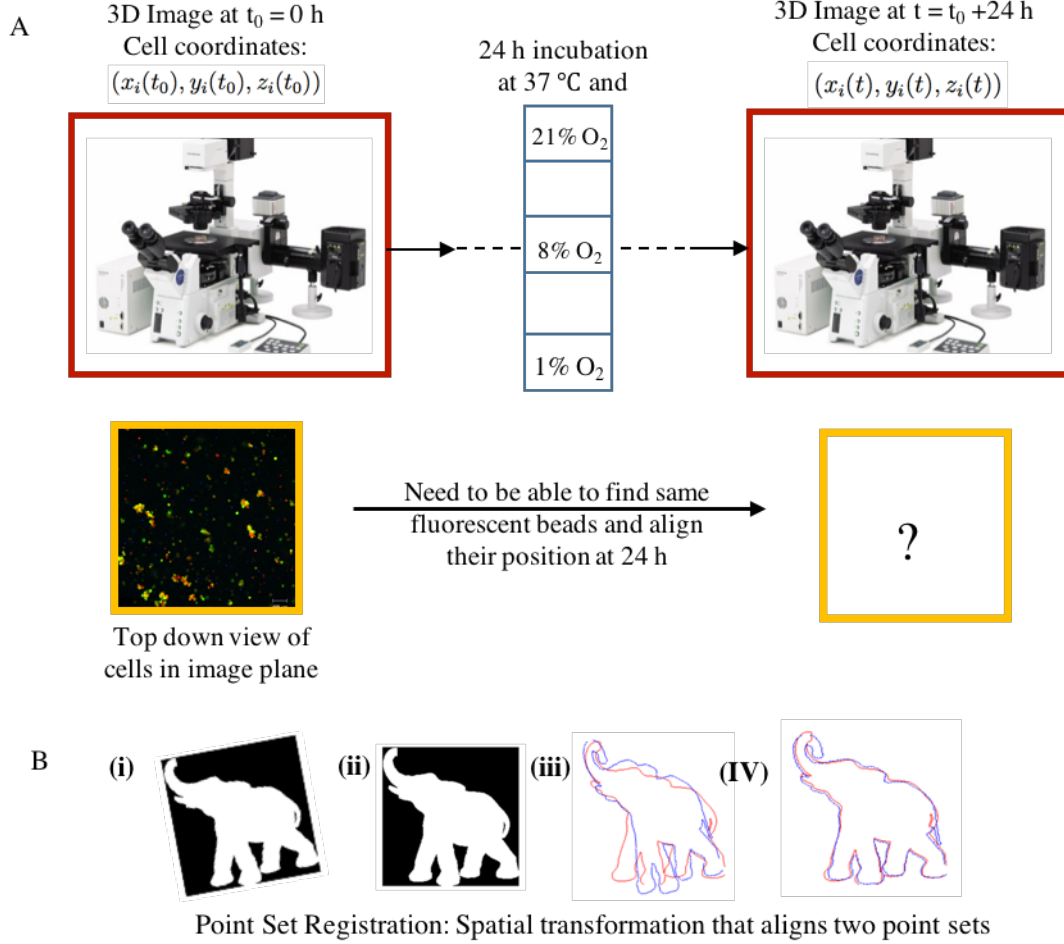


Figure 5.3: **Point Set Registration.** A 3D image is taken at 0 h and the culture dishes are placed in 21, 8 and 1% O₂. A) The challenge is to find the same cells at 24 h. Coherent point drift algorithm is used, which finds the spatial transformation that aligns two point sets. The two point sets are immobile fluorescent beads. B) CPD is illustrated in (i) which is a 2D image, (ii) is the same image rotated, (iii) is an overlay of the outline of both images and (IV) is the result after point set registration. This example is taken from [Du et al. \(2010\)](#).

5.3.3.4 Coherent point drift validation

Before CPD analysis was run on FB, the algorithm was validated on ‘fake points’ generated by the CPD package on python. The initial points are shown in Figure 5.4A, titled ‘position 1’ and are unitless. The points were moved in the x, y, z (0.3, -0.1, 0.2) direction, scaled by 0.9 and 2 out of the 10 points were deleted. This movement is shown in Figure 5.4B and titled, ‘position 2’. The CPD alignment was run and the algorithm correctly aligns points in position 2, overlaying onto the original points in position 1, which is represented by a cross (Figure 5.4C).

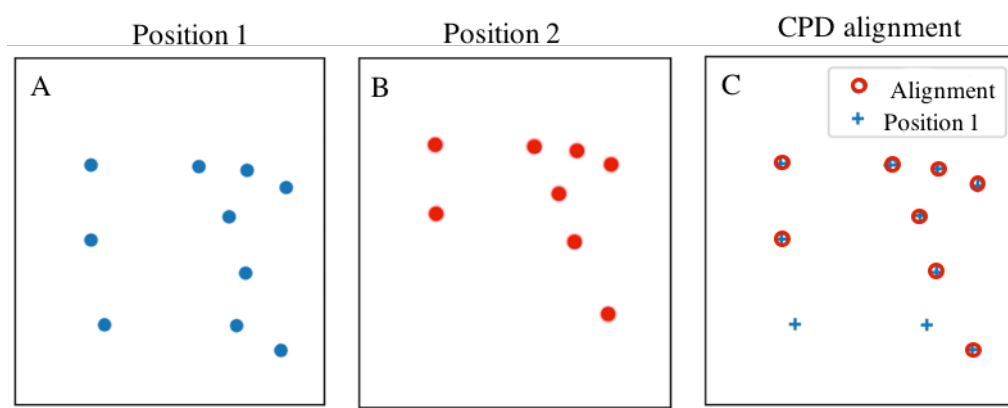


Figure 5.4: **Validation of CPD algorithm.** Ten ‘fake points’ generated with (A) initial points in position 1, (B) moved in the x,y,z direction, scaled and 2 points deleted to give coordinates in position 2. C) The alignment by the CPD algorithm of position 2, to position 1.

5.4 Results

5.4.1 Single cell HLA-G+ population

EVT cells were isolated from first trimester placental samples as described in the materials and methods, chapter 3.2.1. Flow cytometry was used to confirm the presence of the extravillous phenotype, identified using the HLA-G antigen. The percentage of HLA-G+ cells increased from 39.8% on day 0 before plating the cell isolate on fibronectin to 90.2% after overnight incubation (Figure 5.5).

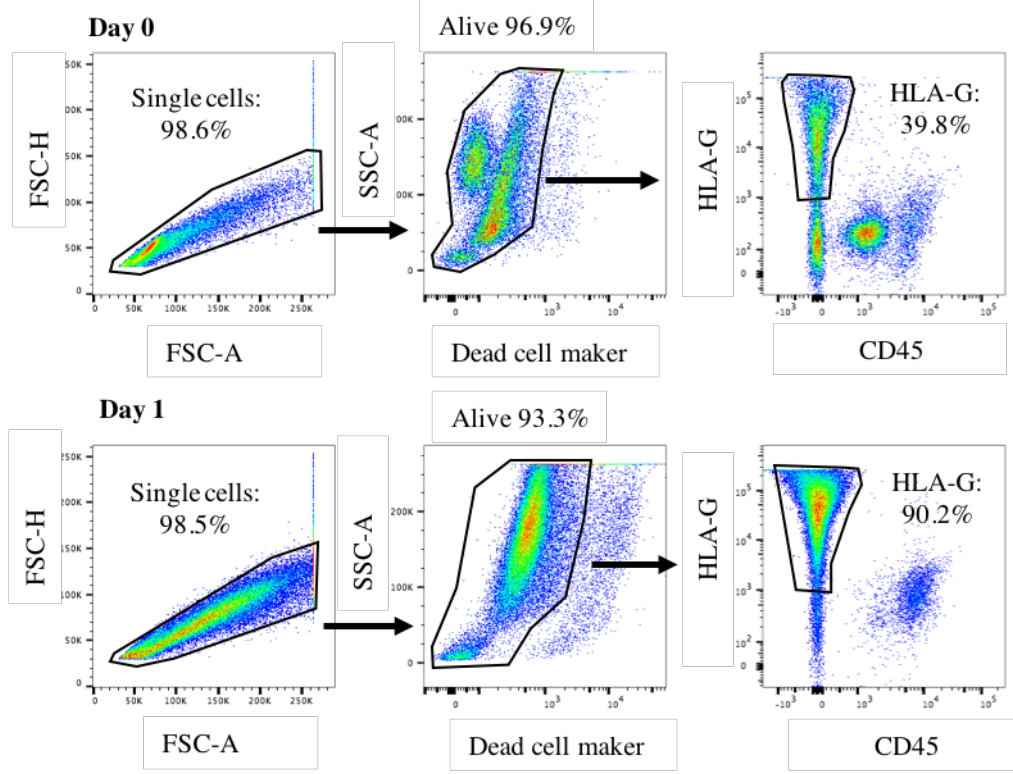


Figure 5.5: **Flow cytometry to confirm purity of HLAG+ EVT cells.** FACS cytometry on sample R067 at 8 weeks gestation. Purity of HLA-G+ cells increases after overnight adhesion onto fibronectin from 39.8% to 90.2%. CD45 is a marker for lymphocytes.

The cell isolates from first trimester placenta samples were found to be prone to clumping, when stained with fluorescent dye in 3D, encapsulated inside Matrigel (Figure 5.6A). This is most likely caused during seeding of the cell isolate onto fibronectin coated dishes. Cells are seeded at a high density to favour EVT binding to fibronectin over the proliferation of mesenchymal cells, when space is available. As a result, EVT adhere in close contact with each other, in a cobble stone morphology (Figure 5.6B). Several techniques were tested to disassociate cells from each other, with the average of two choriocarcinoma cell line JEG-3 populations, which models the extravillous phenotype and one primary isolate shown in Figure 5.6C. The percentage of single cells from the total was determined by taking a sample from each condition and counting by staining with

trypan blue using a hemocytometer. Mechanical agitation performed by cutting the end off a 1 ml pipette tip then resuspending 60 times, together with filtration of the cell isolate through a 40 μm cell strainer was found to increase the percentage of single cells. ACCUMAX, a cell dissociation solution composed of proteolytic, collagenolytic and DNase enzymes had no effect on cell dissociation. As a result, mechanical separation followed by filtration is used in all subsequent experiments with primary cells. The single cell population was confirmed after cells were encapsulated in Matrigel and stained with CellTrackerTM Orange CMTMR. From three primary samples (R001, R002, R003), the mean single cell population was 82.3%. The result can be visualized in 3D with primary cells in red and B221 cells, which do not clump, in blue (Figure 5.6D).

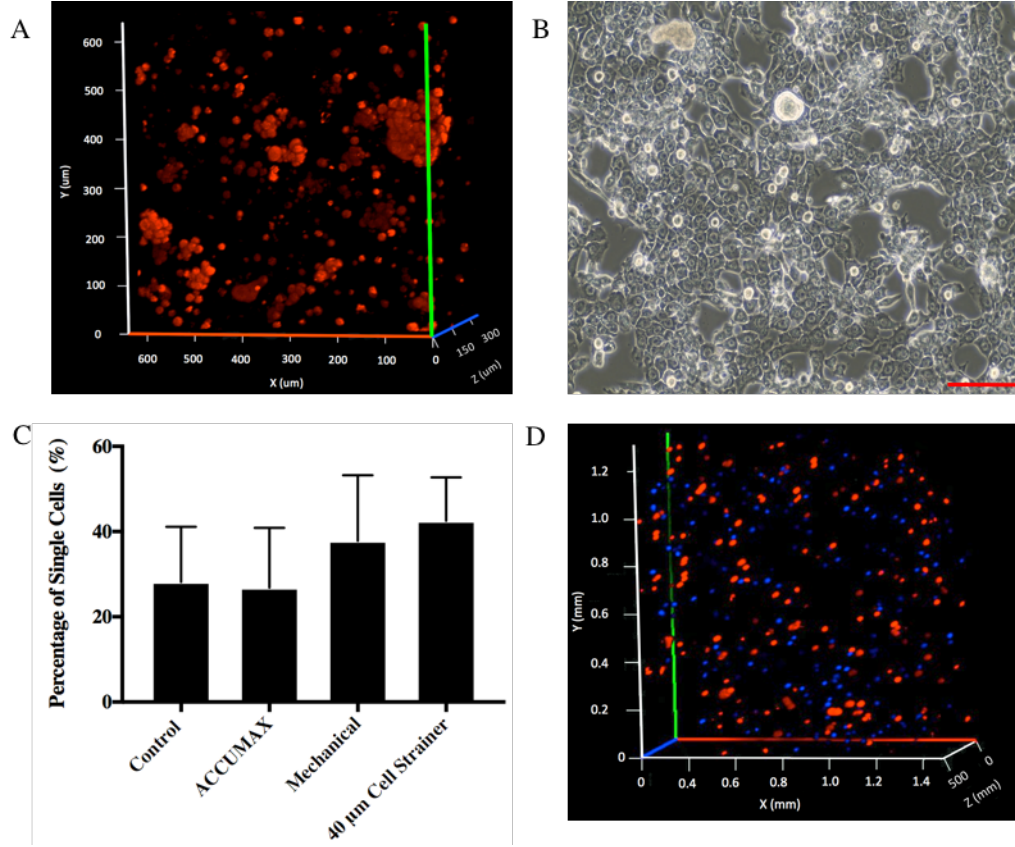


Figure 5.6: **Single cell primary isolate.** Primary cells are prone to clumping together, imaged in (A) 3D, with cells encapsulated in Matrigel and stained with CellTracker™ Orange CMTMR, (B) on fibronectin coated culture dish. C) Mechanical separation by pipetting cell suspension 60 times and passing the isolate through a 40 μm cell strainer was found to increase the percentage of single cells (from the total number of cells) compared to the control. The result of mechanical separation followed by passing the cells through a 40 μm cell strainer is shown in (D) 3D with primary cells (red) encapsulated with B-221 cells (blue), which do not clump. B-221 cells are stained with CellTracker Blue CMAC. Scale bar: 100 μm.

The extravillous phenotype of trophoblast cells can be confirmed visually by staining for the HLA-G antigen. The percentage of HLA-G+ cells was found to vary across the cell isolate of primary samples. From 90.2% as shown in Figure 5.5 to 40% determined using immunofluorescence. This variation can lead to a

wide range of false positives between experiments. Immunofluorescence staining at the end of a migration experiment and after fixation has been demonstrated (Abbas et al., 2017). However, this only confirms the percentage of HLA-G+ cells and does not allow selection of positive cells for analysis.

A protocol has been developed to select for HLA-G+ cells during live imaging and post-experiment analysis. FITC conjugated HLAG antibody, G223 (Figure 5.7A) was incubated with primary cell isolate in PBS without calcium and magnesium for 15 min at room temperature. A 1:40 dilution of G223 was found to give the best signal to noise ratio for at least 24 h, determined by antibody titration. After incubation, the cell suspension was washed three times to reduce background noise. Figure 5.7B is a slice taken from a 3D image and shows positive HLA-G staining. All cells are stained with CellTracker™ Orange CMTMR, with fluorescent beads in blue.

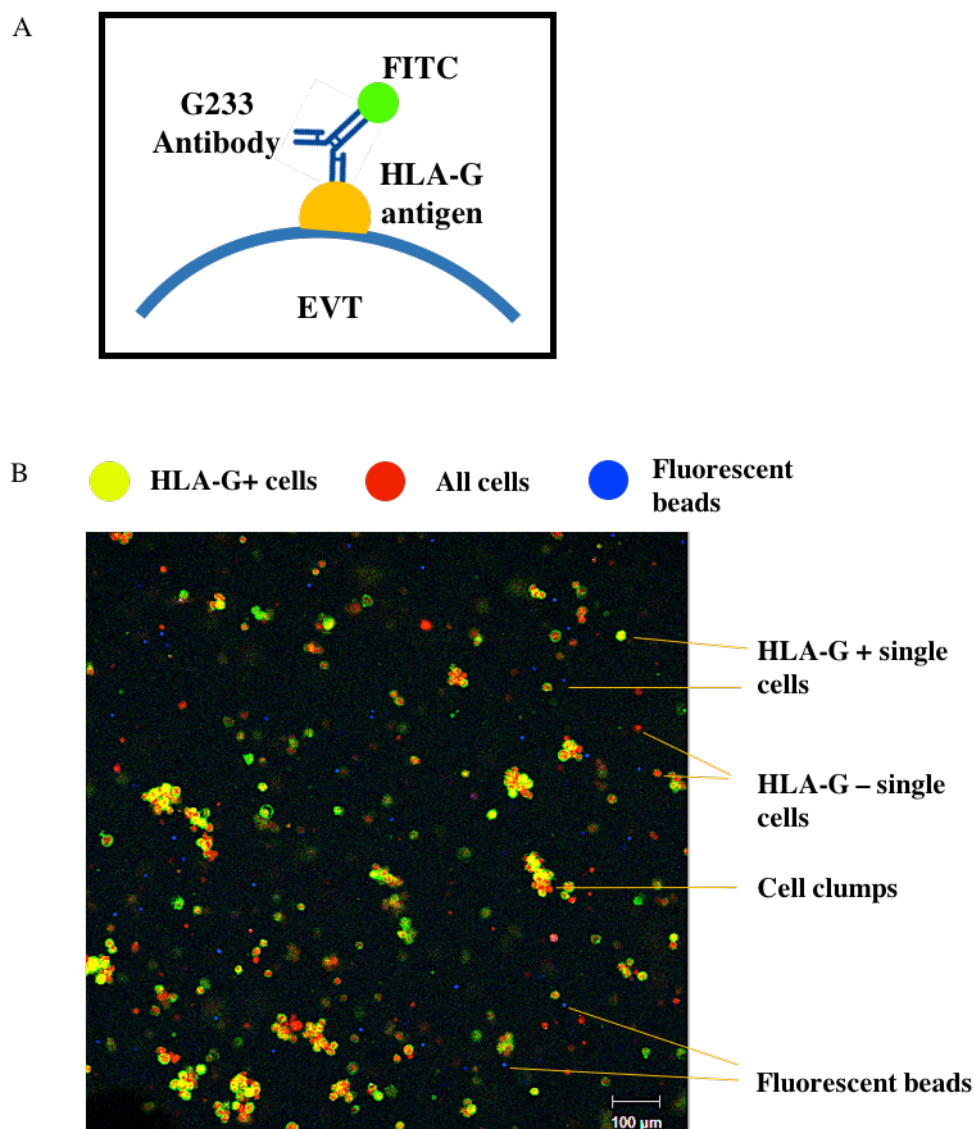


Figure 5.7: **Confirmation of HLA-G⁺ cells during live imaging.** A) FITC conjugated HLAG antibody (G223) was incubated with primary cell suspension to confirm extravillous phenotype. B) A slice through a 3D z-stack was taken using a ZEISS LSM700 confocal microscope and shows the presence of HLA-G positive cells in FITC. All cells are stained with CellTracker™ Orange CMTMR and fluorescent beads in blue.

5.4.2 Coherent point drift analysis

Fluorescent beads with a diameter of 5 μm were added at a concentration of approximately 240 beads per 1 μl of Matrigel/cell solution. The concentration was optimized to ensure there was a wide distribution of beads through-out the volume of the gel. Images with a length, width and height of 1278, 1278 and 805 μm were taken with a LSM confocal microscope. To test the CPD algorithm, a 3D image of the gel containing only FB was acquired and then the culture dish was taken out of the microscope stage and the microscope was switched off and on again. This resets the stage position with the same region of the gel identified by markings on the culture plate and a second image was acquired. Figure 5.8A is an image of all FB projected in 2D with the red circles indicating positive identification of the beads. CPD analysis first matches the same FB from position 1 with those in position 2 (Figure 5.8B). The algorithm was able to match 92% of the beads. The output is a vector of the average shift of the beads in the x, y and z directions and the rotation of the image axis. Figure 5.8C is an example of the CPD analysis with primary tissue (patient R001) for FB imaged at 0 h (position 1 in blue) and 24 h (position 2 in red) at 1, 8 and 21% O_2 .

There are FB missing in the image at 24 h compared to 0 h, which is probably due to weakening of the fluorescence signal from photobleaching. It is possible to reduce the thresholding when identifying the beads but this would increase the noise to signal ratio. Therefore, it is important to ask, how robust the CPD analysis is and whether the results are still valid if there are points missing in position 2.

5.4.2.1 CPD robustness test

To test the robustness of the CPD analysis, 30 single cell coordinates at 0 h and 24 h were generated to produce a RMSD, which follows a Gaussian distribution at the mean. The mean RMSD (28 μm^2) matches that of the RMSD found in section 5.4.3 for EVT at 21% oxygen saturation. This is the ideal case, where the CPD analysis results in 67% of the observations falling within 1 standard deviation of the mean and 100% of observations falling within 2 standard deviations of the mean. Any change from an increase in missing points (MP) in position 2 should

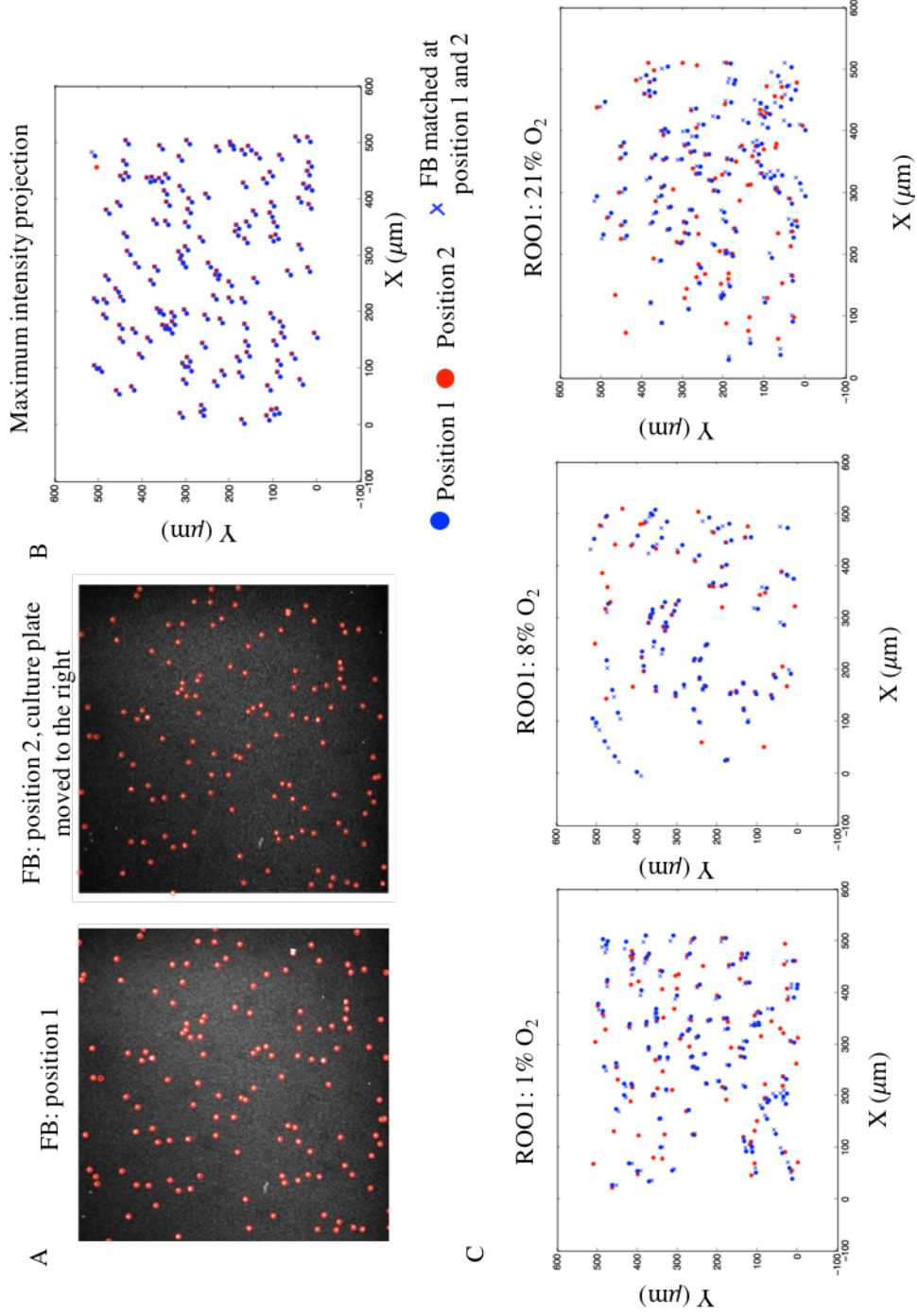


Figure 5.8: **Demonstration of coherent Point Drift analysis.** A) Projected 3D image in 2D of only FB. After the first image was taken the microscope was switched off, culture dish was taken out and microscope was switched on again. This resets the stage position, and the same position was estimated using markings on the dish and a second image taken. B) CPD analysis was run and matches FB from position 1 and 2. C) An example of CPD FB matching with the patient sample R001 at 1, 8 and 21% oxygen.

result in a change in the distribution of the density curves and mean RMSD. The same FB as in Figure 5.8A are used with 20, 40, 50 and 60% of the points in position 2 deleted randomly (Figure 5.9A). The CPD analysis is run with the MPs, which produces an output vector field of the shift of the beads in x, y and z and the rotation of the image axis. The vector for each of the MPs is applied to cell coordinates at 24 h.

Next, given the large depth (805 μm) that was imaged, the signal-to-noise ratio was found to decrease as the distance increases from the objective. Therefore, it is important to ask whether CPD analysis is possible, if only a proportion of the z-stack is analyzed. For this question, CPD analysis was done on the first 10% of the z-stack depth (Figure 5.9D).

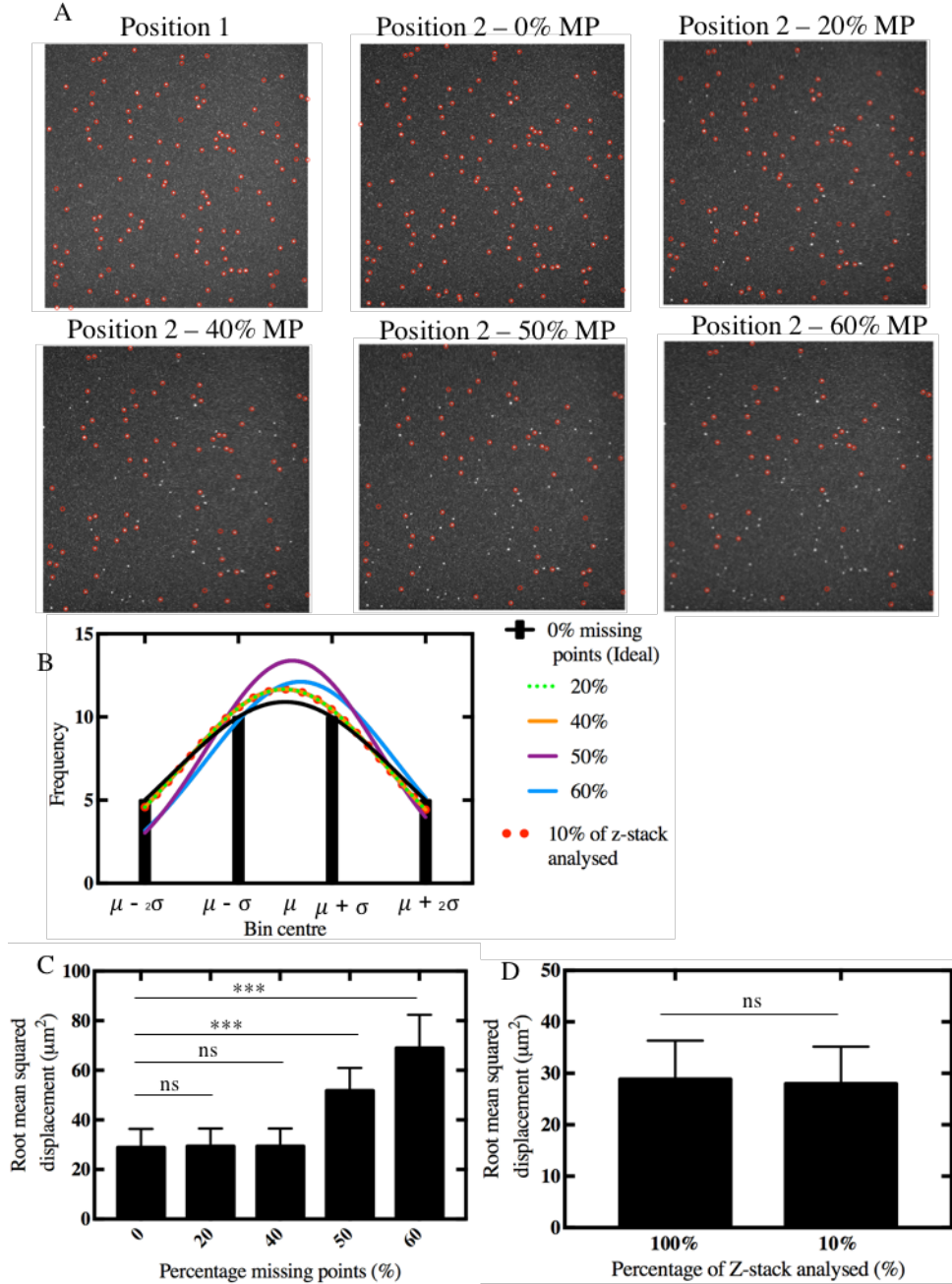


Figure 5.9: **CPD robustness test.** A) Points from position 2 were deleted at random so that 20, 40, 50 and 60% of points were missing. B) Density curves from CPD analysis shows deviation from the mean. C) There was a significant change in RMSD when 50 and 60% of points were deleted. There was no significant change in RMSD, when CPD analysis was done on the first 10% of the z-stack compared to 100% of the image depth. Mean $\pm$ SD, *** $P < 0.001$, ns - not significant, determined using unpaired t-test.

There was no significant change in RMSD when 20 and 40% of the points are deleted compared to the ideal (0% MP). The distribution can still be described as Gaussian, although it is not a perfect fit (Figure 5.9 B&C). For 50 and 60% MPs, the mean increases linearly, which was statistically significant and 40% should be considered the maximum in CPD analysis. When only 10% of the z-stack was analyzed, there was no significant change in the mean RMSD and the distribution of means can be described as Gaussian (Figure 5.9D).

5.4.3 EVT motility

EVT from six primary human placental samples of gestational age between 7-9 weeks were isolated as described in materials and methods, chapter 3.2.1. For each oxygen condition the RMSDs of 30 HLAG+ cells were analysed. The same HLAG+ cell at 0 h and 24 h was identified using FB as reference markers. Only single HLAG+ cells were chosen for analysis as migration dynamics have been shown to differ when cells are in contact with one another (Friedl and Gilmour, 2009). Cells were assumed dead if the HLAG+ FITC signal had weakened significantly and when the fluorescent signal appeared granulated. Cells that had negligible RMSD were discounted for analysis and were assumed dead. The data was found not to follow a gaussian distribution using the D'Agostino & Pearson normality test. Therefore, non-parametric tests were used to assess statistical significance. The RMSD of HLAG+ EVT cells was found to be larger under low oxygen compared to 8% and 21% oxygen concentration, but this increase was not statistically significant (Friedman test, $P = 0.1416$, multiple comparison test, $P > 0.05$, Figure 5.10 A&B). Linear regression and Pearson correlation analysis were performed with a R^2 and R value of 0.88 and -0.93 respectively suggesting there is a linear relationship with motility increasing as oxygen decreases. However, evaluating individual samples, this trend is only seen with P065 and R001 over the three oxygen values. Interestingly, a wide distribution of RMSD was found in this experiment. Possible explanations include, cells that are at different stages of the cell cycle or a subpopulation of more migratory EVT cells.

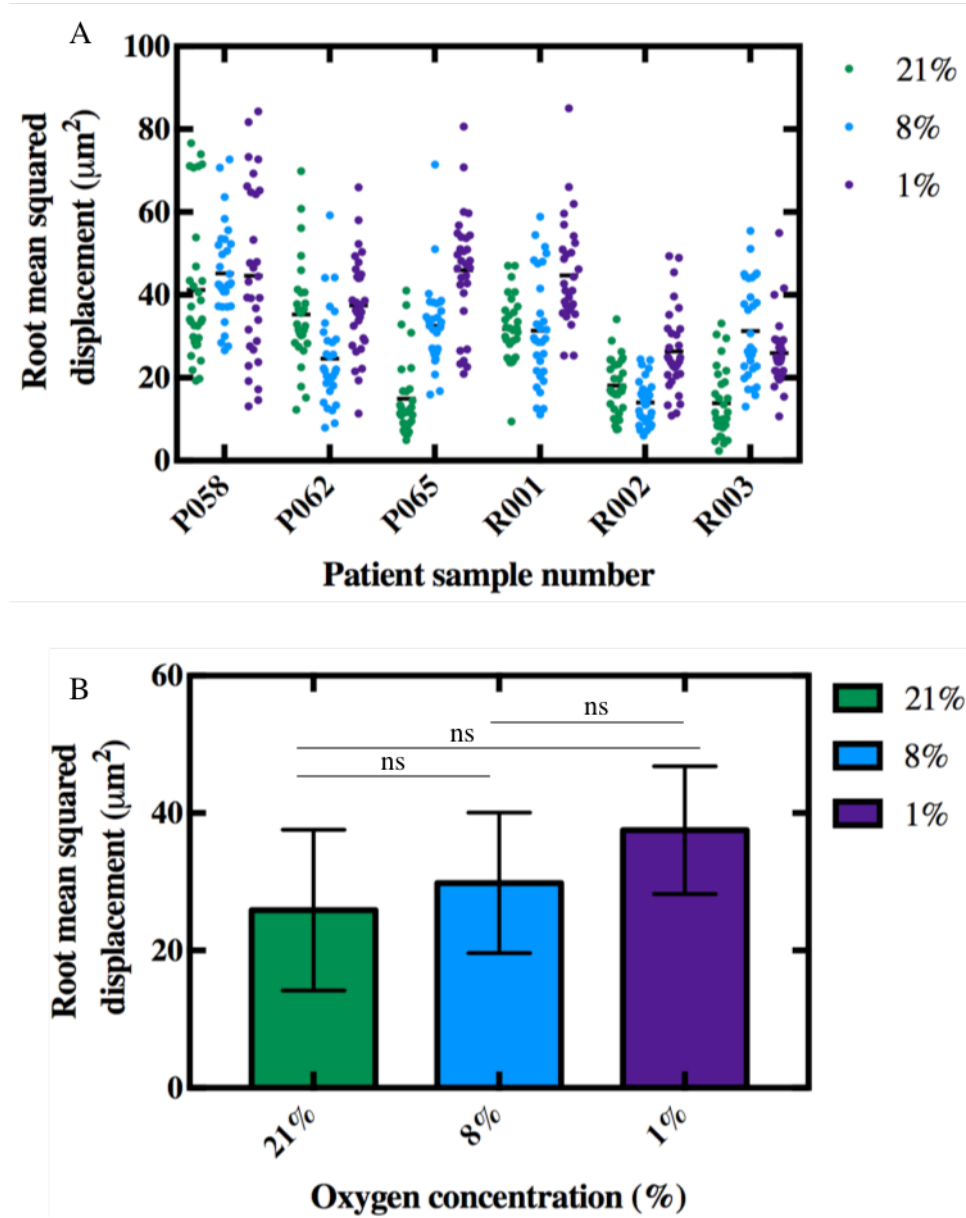


Figure 5.10: **EVT motility** A) The RMSD of HLAG+ EVTs from six primary samples at gestational age 7-9 weeks was estimated. Mean $\pm$ SD B) EVT cells were found to be more motile under 1% oxygen concentration compared to the control 8% and 21%. This difference was found not to be statistically different. Mean $\pm$ SD, ns= not significant with $P > 0.05$, determined using Friedman and Multiple Comparison test.

5.4.3.1 EVT cells in 3D do not follow random walk

Next, it is important to ask how the RMSD measured over two time points, compares to RMSD for cells imaged using time-lapse continuous imaging. The RMSD was quantified for HLAG+ EVT cells imaged every 20 min for 6.7 h at 21% oxygen. The cells were embedded in Matrigel, in 3D and placed in an environmental chamber at 37 °C, which fits around the confocal LSM microscope. In 6.7 h, 20 images were taken with 32 cells in each image. The mean RMSD over 6.7 h for patient sample M76 was $9.7 \mu\text{m}^2$, which was $16 \mu\text{m}^2$ lower than the average of six samples over 24 h (at 21% oxygen), but in the same order of magnitude (Figure 5.11A). Four cells had negligible RMSD for three consecutive images and so were assumed dead and discounted for analysis (figure 5.11B). A wide distribution of RMSD was found, which suggests cell health was heterogeneous or that the cells are at different stages of the cell cycle and they migrate accordingly (Figure 5.11C). The RMSD was found to plateau within 20 minutes of imaging, which suggests EVT motion was constrained in 3D space and does not obey random walk (Figure 5.11A). Figure 5.11D is a 4D map of a representative cell, moving in 3D space over 6 h. From this result, EVT motion in 3D can be described in terms of the volume explored, rather than a direct measurement of migration, as there is no way to quantify how quickly the plateau in RMSD is reached in low oxygen.

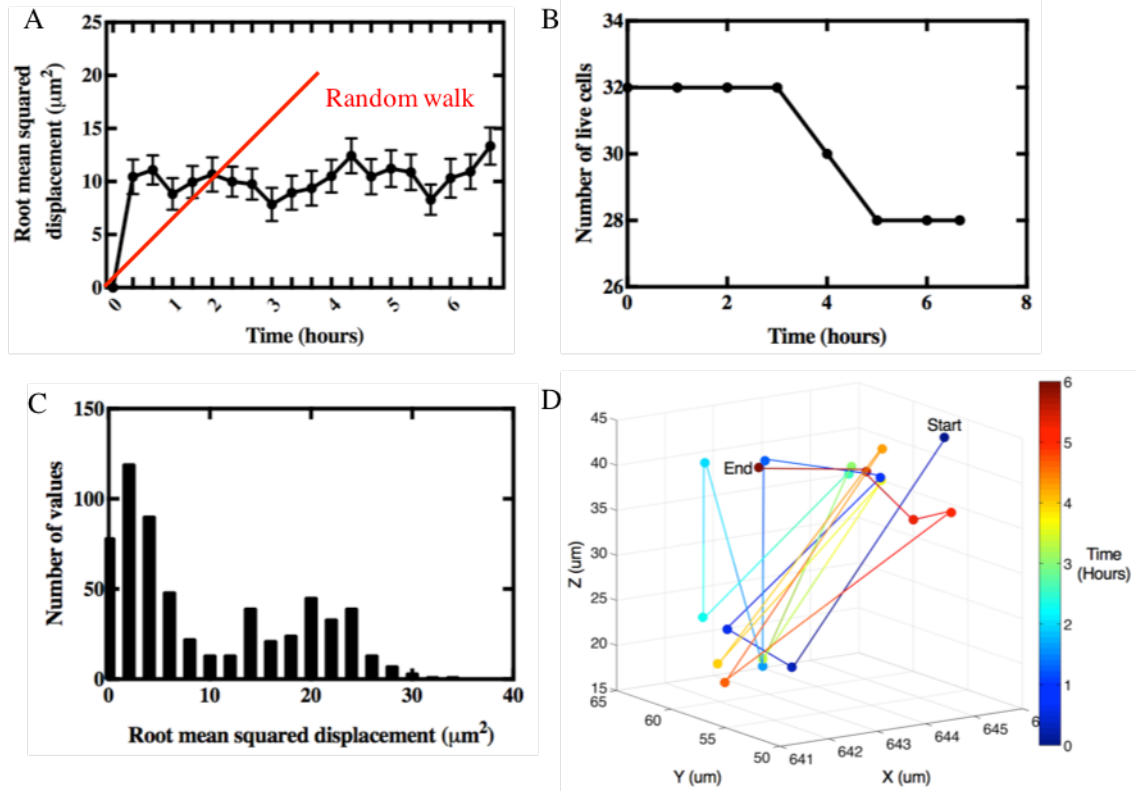


Figure 5.11: **RMSD from time-lapse HLAG+ EVT cells.** A) The RMSD of 32 HLAG+ EVT cells was estimated when imaged using time-lapse microscopy for 6.7 h every 20 min. The RMSD was found to plateau, suggesting EVT motion was constrained and does not obey random walk in 3D. Random walk would result in a linear trend of RMSD over time, as indicated with the red line. B) 4 cells were assumed dead after no motion from three consecutive images and discounted from analysis. C) EVT motion does not follow a Gaussian distribution. D) A 4D map of a representative cell in 3D over 6.7 h.

5.5 Discussion

5.5.1 Simple and economical technique for isolating single HLAG+ EVT

Reliable models to study trophoblast invasion *in vitro* are lacking, with increasing evidence that current trophoblast cell lines differ substantially at the transcript level (Apps et al., 2011). EVT isolated from primary tissue is best for studying invasion but challenges exist with producing a high purity HLAG+ single cell population. Two simple techniques performed consecutively, mechanical agitation of the cell isolate and then passing the isolate through a 40 μm cell strainer produced a population of greater than 80% single cells. Confirmation of the presence of the HLA-G antigen during live imaging allows for easy selection of the extravillous phenotype during analysis, which is not possible if the sample is fixed and stained after an experiment. HLA is not involved in the migration/invasion pathway of EVT cells, as the system is responsible for the regulation of the immune system (Chaplin, 2010). Other techniques are available for single cell sorting such as fluorescence-activated cell sorting (FACS) or magnetic separation using magnetic nanoparticles. However, FACS may inadvertently lead to compromised cell viability and yield (Basu et al., 2010). The technique described in this chapter, is simpler and economical.

5.5.2 The role of low oxygen in EVT migration/invasion

The method used to quantify motility attempts to overcome some of the challenges in this field. The use of single HLAG+ cells cultured for 24 h and a 3D matrix are advantages of this method. The small volumes used and the gradual reduction in oxygen for the low oxygen condition, allows for rapid equilibrium and protects the cells from ischemia-reperfusion injury. Point set registration using CPD analysis is simple and robust, enabling quantification of cell motility over 24 h, when cells are placed in oxygen-regulated incubators. A trend of increased motility with low oxygen was found, but this trend was not found for all samples and was not statistically significant. Intersample variation is expected with primary samples, with sample health and time from surgery to cell

isolation important factors. Nevertheless, from these results it is not possible to make a definitive conclusion on the effect of low oxygen on trophoblast motility. However, these results open the debate that oxygen may have no effect on trophoblast motility. This may explain the number of conflicting studies and why this question is yet to be answered. Certainly, techniques such as microfluidics to investigate cell motility under directed oxygen gradients developed in chapter 6 will add to the debate. In addition, CRISPR/cas9 to knock down genes associated with oxygen sensing and migration such as HIFs and MMPs will be essential.

EVT motility was measured as RMSD, which is the average distance a cell moves in a given time interval, not the total distance travelled. It is important to ask, how good the use of two time points, at 0 and 24 h, is at estimating RMSD. The RMSD at the same oxygen concentration, 21% over a 6.7 h time-lapse recording was in the same order of magnitude as the experiment over 24 h. Although, these are two different time intervals, because RMSD was found to plateau immediately, suggesting EVT motion is constrained in 3D, it is assumed the RMSD will not change if the time-lapse recording was extended to 24 h. Looking at individual samples, three were within 10 μm^2 and one within 5 μm^2 of the time-lapse over 6.7 h. Variability in RMSD is expected as there is wide genetic variation in humans that could influence EVT behaviour.

The finding that EVT motion in 3D is constrained and does not obey random walk, is in line with the finding of Wu *et al.*, who hypothesised that 3D cell migration is influenced by heterogeneity in the density, pore size, fibre thickness and matrix stiffness of local ECM (Wu *et al.*, 2014). In this study, EVT cells were found to move back and forth, occupying the same space in 3D. One explanation for this behaviour could be the lack of matrix metalloproteinases (MMPs) production, which are a group of enzymes that break down matrix proteins. Cell migration through an extracellular environment of pore size smaller than a cell is known to depend on MMPs (Zaman *et al.*, 2006). Recently, Majali-Martinez found that low oxygen abolishes the effect of endothelin-1 (ET-1), a vasoconstrictor peptide, which is known to down-regulate MMP14 and MMP15 production in primary EVT cells (Majali-Martinez *et al.*, 2016). In the absence of external stimuli such as a cytokines and at atmospheric oxygen, the pathway for MMP

production may not be activated sufficiently. If low oxygen does regulate EVT invasion *in vivo*, MMPs will be part of the pathway, hence this may be why low oxygen is shown to increase EVT motility in this study compared to previous, albeit this increase was not significantly different. The cells may be less constrained and at 1% or 8%, their motion may be more like random walk. Although MMPs have been measured in studies looking at low oxygen and trophoblast invasion, these have been in 2D or explant tissue. Therefore, there is a need to investigate MMP production under low oxygen in primary EVT and in 3D.

5.5.3 Limitations of experiment

In this study, EVT motility was quantified from cell coordinates at 0 h and 24 h and the RMSD was determined. RMSD is not a measure of velocity and continuous imaging at a higher temporal resolution (minute scale) would be needed to estimate this parameter. In order to extend this study, more time points are needed to determine the path a cell takes, change in migration throughout time and directional persistence of the cells. In addition, quantification of changes in gene expression would offer explanation to why cells are more motile under low oxygen. It would be useful to determine a time-dependent relationship of the up-regulation of hypoxia-inducible factors (HIF) at oxygen concentrations between 1-8%.

Finally, it is important to recognise the limitations of *in vitro* experiments with primary cells at low oxygen. EVT cells go from $\sim 2.6\%$ (Jauniaux et al., 2000) in the uterus, to 21% after the termination then back down to low oxygen for the experiment. This process will induce stress in the cells leading to gene expression changes and potentially the production of reactive oxygen species (Halliwell, 2003; Gorr, 2017). Because of this oxygen was reduced gradually over 3 h from 21% to 1%. However, more work is needed to understand the effect of stressed induced genes on the mechanisms that regulate cell migration. To mitigate stress from hyperoxia and hypoxia, a range of technologies such as gas-tight boxes (for tissue transport) and gas-controlled incubators could be used (Yazdani, 2016).

5.6 Conclusion

This chapter demonstrates the challenges of investigating low oxygen and trophoblast invasion. It addresses a number of the limitations of previous studies with the use of primary EVT cells and analysis in 3D. A trend of increased motility was found under low oxygen but this was not found to be statistically significant. Through the use of low volume culture dishes, equilibrium of oxygen is rapid and no medium change is required during incubation, avoiding ischemia-reperfusion injury. HLAG+ EVT cells were found to be constrained in 3D space and do not follow random walk. The production of MMPs is hypothesised to influence migration of HLAG+ EVT cells in 3D. Future migration experiments in low oxygen should focus on a comprehensive gene expression study on primary HLAG+ EVT cells, to measure markers of hypoxia and MMP production. These experiments should be conducted in 3D.

Chapter 6

Microfluidics for the study of oxygen gradients at the maternal-fetal interface

The effect of low oxygen on trophoblast motility was examined in the previous chapter. In this chapter, a microfluidic based system to study whether extravillous trophoblast migration is directed in three-dimensions (3D) is developed. The system is validated with optical based oxygen sensors and COMSOL computational modelling. The chapter concludes with a list of troubleshooting measures to aid future users on the techniques discussed.

6.1 Introduction

Trophoblast invasion is spatially and temporally controlled during the first trimester of pregnancy. Oxygen is thought to be one of the regulators, in part because of the decreasing oxygen gradient from the decidua towards the developing embryo (Graham and Burton, 2004; Huppertz et al., 2009). The trophoblast cell column is at the border between the low oxygen placenta, with the distal ends in contact with the decidua at a higher oxygen concentration (Huppertz et al., 2009). This is the location from which extravillous trophoblast (EVT) invasion takes place. The oxygen gradient exists when invasion is most prominent, from 20 mm Hg (2.5%) at the intervillous space to 60 mm Hg (8.4%) at the decidua (Figure 6.1A). Oxygen at the intervillous space increases to 60 mm Hg (8.4%) with the arrival of maternal circulation at the end of the first trimester (Figure 6.1B) (Jauniaux et al., 2000). The question therefore arises, is EVT migration directed along the path of increasing oxygen?

Most current *in vitro* assays for studying cell migration are end-point, unsuitable for primary cell work, and allow control of chemical factors only. Any assay needs to be 3D, and the oxygen gradient needs to be relevant on the scale at which cells can sense changes in the local oxygen concentration. In recent years, microfluidics has emerged as a new platform for the study of cell migration, as it allows detailed control of a number of relevant physical and chemical properties (Sung et al., 2011; van Duinen et al., 2015). In this chapter, an *in vitro* model with an oxygen gradient that approaches the gradient during the first trimester of pregnancy is designed and validated, using microfluidics.

6.1.1 Methods to study EVT cell migration

Conventional methods to study trophoblast invasion both *in vivo* and *in vitro* have significant drawbacks. There are marked differences in the placentation of laboratory animals when compared to humans, with the deep interstitial invasion characteristic of humans only found in the great apes (Moffett and Loke, 2006). *Ex vivo* explants of placentas suffer from poor viability, and sampling across the whole placenta is difficult (Ashton et al., 2004). Existing *in vitro* methods include the Transwell assay where cells are placed in an insert and migrate through

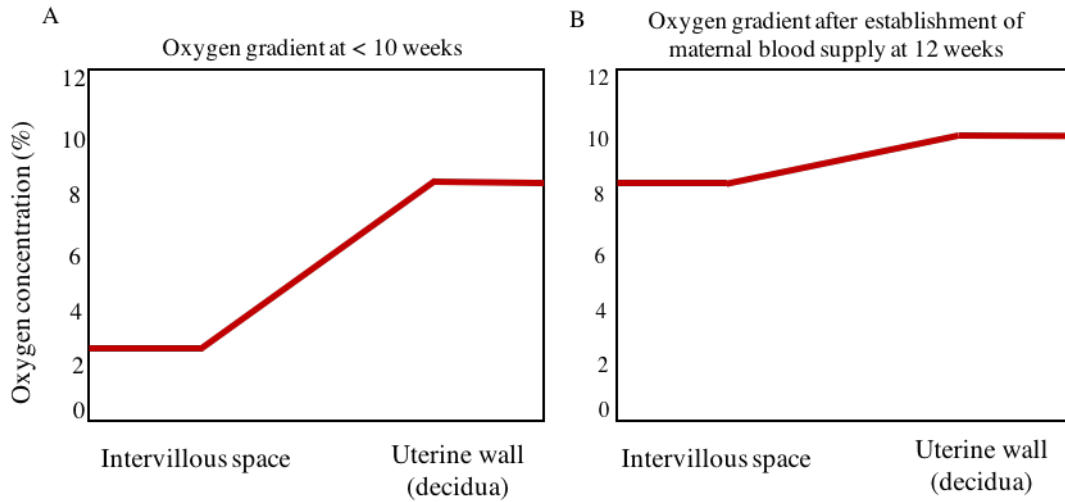


Figure 6.1: **Oxygen gradient at the maternal-fetal interface.** *In vivo* measurements prior to termination of anticipated normal pregnancies using oxygen electrodes (A) prior to 10 weeks gestation and (B) weeks including 12 to 15 (Jauniaux et al., 2000).

a cell permeable membrane towards a chemoattractant (Justus et al., 2014). Alternatively, in the scratch assay, a gap is created by ‘scratching’ a monolayer of cells and the migration rate is determined by time lapse microscopy (Jonkman et al., 2014). These *in vitro* assays have two main drawbacks. First, they are difficult to use with primary cells because large numbers of purified trophoblast cells from first trimester placentas are needed. Second, they are not a measure of true chemotaxis, as analysis of cell migration in two dimensions neglects the importance of the 3D environment in which the cells find themselves *in vivo*, and hence has little physiological relevance (Roussos et al., 2011; Katt et al., 2016).

In contrast to these existing migration assays, microfluidic devices allow the precise control of chemical and physical gradients in a 3D environment (van Duinen et al., 2015). Cells are embedded in a physiologically relevant hydrogel matrix, and single cell migration is observed in real time under constant fluid flow in 3D (Shin et al., 2011). Individual cell migration tracks can be quantified, and additional migration characteristics such as migration speed and directionality can be obtained (Polacheck et al., 2013). Importantly, because only a few

6.2 Current approaches to oxygen control in microfluidics

thousand cells are required, this assay can be readily and repeatedly performed using primary trophoblast cells. This compares to 1.9×10^5 cells required at confluency for the scratch assay in a 24-well plate (Jonkman *et al.*, 2014; Corning, 2017). In this chapter, an oxygen gradient is established within a microfluidic device to study trophoblast migration in 3D and in real-time.

6.2 Current approaches to oxygen control in microfluidics

There are two main strategies for generating an oxygen gradient: (1) chemical reactions that generate and scavenge oxygen (2) feed of low oxygen gas or liquid. The high diffusivity of oxygen in PDMS, the primary material used to manufacture microfluidic devices, allows for fast diffusion of gas molecules between channels (Cox and Dunn, 1986). An example of the first strategy is a study by Chen *et al.*, who cultured cells between two channels that generate and scavenge oxygen (Chen *et al.*, 2011). The main limitation here is the use of chemicals that are acutely toxic if inhaled or in contact with skin, and that are corrosive to plastics and metals. Because microfluidics is prone to fluid leaks from fittings, this route was deemed not safe and a risk to microscope equipment. The second route is use of low oxygen gas in a supply channel adjacent to the channel with cells. This is the most common platform reported in the literature, but the set-up is complex with the need for bulky pressurised gas bottles and gas flow on a time scale of hours (Ungerböck *et al.*, 2013). Thomas *et al.*, simplified this with oxygen control delivered with liquid pre-equilibrated to low oxygen by diffusion (Thomas *et al.*, 2011). However, this set-up still requires a controlled gas composition, and therefore different gas bottles have to be used for on-chip control of oxygen. In order to overcome these limitations, the set-up has been simplified further, removing the need of a controlled gas composition, instead oxygen in the microfluidic chip can be controlled by flow rate of liquid into the device. The aim is to produce an economical, simple and safe method for oxygen control.

6.3 Study requirements

There are four main requirements in this study (Table 6.1). First, an oxygen gradient concentration of between 1-3% to 8-10% is required for physiological relevance. *In vivo* sampling using oxygen electrodes determined a gradient of 2.5% and 8.4% between the intervillous space and maternal decidua (Jauniaux et al., 2000). Given there was a range of concentrations sampled, in part because of gestational age and location, there should be flexibility in the concentrations in the microfluidic gradient. Second, the time scale of the experiment should be at least 6 h. In a time dependent study with multiple cancer cell lines, Chi et al., found a 2-3 fold change in mRNA when under 2% oxygen for 6 h (Chi et al., 2006). No similar study has been performed with trophoblasts but to allow time for translation of RNA into protein, an experiment time of 10 h is recommended. Third, there needs to be simple control of oxygen in the device, removing the need for controlled gas compositions. Controlled gas mixtures are expensive to procure and there are restrictions to use of pressurised gas in rooms without windows. Microscopes tend to be enclosed in such rooms to protect samples from light. Finally, oxygen in the system should be monitored and the gradient validated. A stable oxygen gradient is important to limit the stress on cells.

6.3 Study requirements

Requirement	Performance benchmarks
Oxygen gradient concentration	1-3% to 8-10%
Time scale of experiment	10 h
Simple control of oxygen in the device	Remove need for controlled gas composition
Monitoring and validating oxygen gradient	Real-time oxygen monitoring of liquid feed lines and on-chip gradient. COMSOL fluid dynamics to simulate oxygen gradient.

Table 6.1: Requirements for oxygen control in the microfluidic device.

6.3.1 Design of gas-liquid diffusion system

Traditionally, low oxygen work *in vitro* is carried out in incubators or hoods with sophisticated gas control (Brennan et al., 2014). Given the need to monitor EVT migration in 3D, an experimental system needs to be designed that is modular and can be assembled next to a microscope. Such a design can be divided into two parts; (1) a system to control oxygen gas concentrations between 0-21%, and (2) diffusion of this gas mixture to produce pre-equilibrated liquid, which is delivered to the microfluidic device to generate a gradient of oxygen.

6.3.2 Part 1: Gas control

A number of commercial systems were considered to produce oxygen gas mixtures. Most are stand-alone and mix oxygen with nitrogen gas to concentrations between 0% and 20.9%, in 0.1% increments (Baker, 2015). The high cost of these systems (approximately \$8,000) meant one had to be designed and built in-house. Multiple methods were considered to produce a low oxygen gas mixture with simplicity

6.3 Study requirements

and costs being the main requirements. The methods considered were; (1) adding nitrogen to a fixed volume of air, (2) adding oxygen to a fixed volume of nitrogen and (3) mixing a fixed volume of air, oxygen and nitrogen. Options (2) and (3) were discounted due to safety concerns of using pressurised oxygen gas, which is a fire and explosive hazard. Option (1) was chosen as it limits the number of gases in the system, thus reducing need for additional supply lines, flow control and space.

6.3.3 Part 2: Diffusion of gas mixture

Pre-equilibrated liquid is delivered to the microfluidic device using a syringe pump, which drives fluid flow. The gas concentration in this liquid needs to reach equilibrium with the desired low gas mixture. It is not viable to equilibrate this liquid before it is loaded into a syringe, as it would not be in a closed system and the dissolved gas mixture would quickly equilibrate to atmospheric conditions. A sealed vessel was designed to contain the low gas mixture and be the site for diffusion. Liquid is pumped through a coil of gas permeable tubing and equilibrates to the low oxygen concentration. The liquid then exits the vessel but is now contained in gas impermeable tubing to prevent oxygen diffusion from atmospheric gas. The process flow diagram for this design is shown in Figure 6.2.

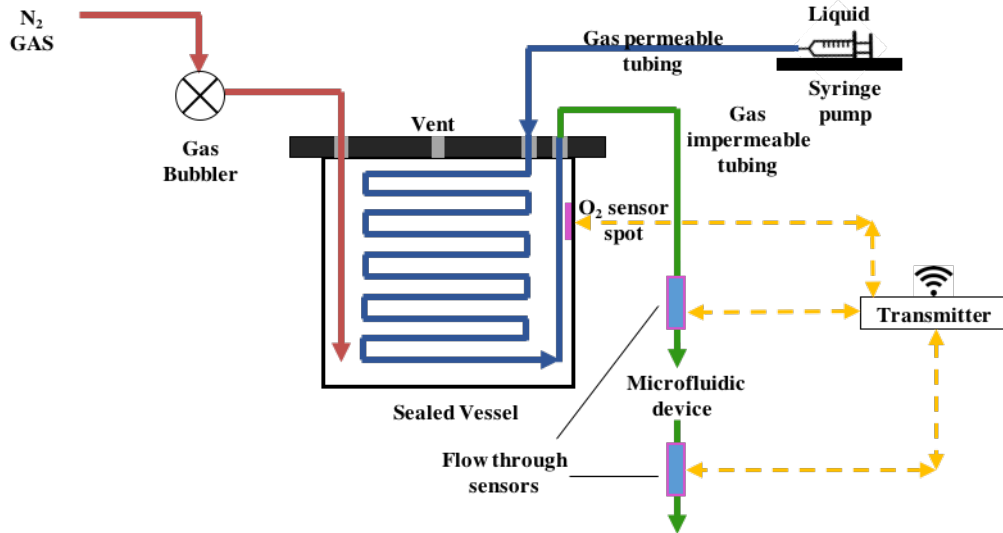
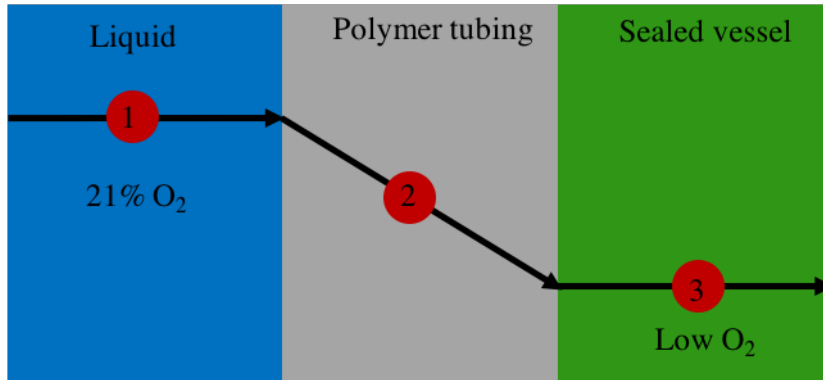


Figure 6.2: **Process flow diagram of gas-liquid diffusion system.** Liquid is perfused through gas permeable tubing using a syringe pump into a sealed vessel, which contains a low oxygen gas mixture. There is a concentration gradient of oxygen between gas dissolved in the liquid and bulk oxygen in the vessel. Oxygen diffuses out of the liquid throughout the length of the coil. The liquid exits the vessel in gas impermeable tubing towards the microfluidic device. The oxygen concentration in the vessel and at the inlet and outlets of the microfluidic device is monitored in real-time using optical oxygen sensors. A 5 mm diameter sensor spot is attached to the inside of the vessel, coated with oxygen sensitive dye. A fibre optic cable excites the dye and in the presence of molecular oxygen, the dye is quenched with the amount of quenching proportional to the oxygen concentration. Flow through sensors at the inlet and outlets of the device measure the dissolved oxygen content in the liquid.

6.3.3.1 Diffusion dependent parameters

There is a concentration gradient between gas dissolved in the liquid and gas in the vessel. Oxygen penetration through polymer tubing occurs in three stages, as shown in Figure 6.3. Oxygen molecules dissolved in the liquid diffuse to the boundary of the liquid and are adsorbed onto the polymer surface. Oxygen molecules then diffuse through the bulk polymer tubing and are desorbed into

the sealed vessel.



1. Adsorption onto polymer surface
2. Diffusion through bulk polymer tubing
3. Desorption into gas sealed vessel

Figure 6.3: **Permeation of gas through polymer.** The driving force is a concentration gradient between oxygen dissolved in the liquid and the bulk gas concentration in the vessel. Oxygen molecules adsorb onto the polymer surface, diffuse through the bulk polymer and then desorb into the sealed vessel.

Oxygen permeation of the polymer membrane depends on diffusion but the nature of the diffusion process depends on the phase of the system, material properties, the size of the permeating molecules and the driving force. Fick's law describes the rate at which a dissolved gas diffuses across a membrane given certain properties of the membrane and gas:

$$V'_{gas} = \frac{DA\Delta P}{\Delta x} \quad (6.1)$$

where V'_{gas} is the rate of gas diffusion across a permeable membrane, D is the diffusion coefficient of a particular gas for that membrane, A is the surface area of the membrane, ΔP is the difference in pressure of the gas across the membrane or the driving force and Δx is the thickness of the membrane. For simplicity, the concentration (C) gradient will be used to describe the driving force across

6.3 Study requirements

the membrane, which is related to pressure by the ideal gas law, $\Delta P = \Delta CRT$, where R is the ideal gas constant and T is temperature.

The aim is to optimise the diffusion process to make it rapid and selective. Whereas the phase of the system (liquid to gas) and size of permeating molecules are fixed, the tubing material and driving force can be optimised. The factors that affect permeability of a polymer are solubility and diffusivity of the permeating molecule and the material chemistry, which includes chain packing and side group complexity, polarity and crystallinity (Ismail et al., 2015). There is little scope to choose a material based on these properties due to the limited information given by manufacturers. However, most manufacturers provide the permeability of oxygen, nitrogen and carbon dioxide gas through a polymer, which is defined as flux over the pressure gradient. Silicone was chosen as the tubing, as it is one of the most permeable elastomers and has excellent biocompatibility (Cole-Parmer, 2017; Bernard and Mader, 2001). Monomers of silicone elastomers are cross-linked and cured into solids, which are then transformed into tube structures with the strength to withstand high pressure flows. A search was conducted and platinum cured silicone tubing was chosen with an oxygen permeability of 7691 ($\text{cc} \times \text{mm}/(\text{cm}^2 \times \text{sec} \times \text{cmHg}) \times 10^{-10}$). To increase the rate of gas diffusion across the membrane, the smallest tube wall thickness was chosen, 0.5 mm. The driving force can be optimised by changing the flow rate of liquid through the sealed container. For liquid leaving the sealed vessel, Viton tubing, which has one of the lowest permeabilities to oxygen was chosen: 13-15 ($\text{cc} \times \text{mm}/(\text{cm}^2 \times \text{sec} \times \text{cmHg}) \times 10^{-10}$).

6.3.4 Strategy for oxygen sensing

Monitoring oxygen throughout the system is important for experiment validation. Optical sensors were chosen as they do not consume oxygen and provide real-time readings every second (Stein et al., 2003). The principle is based on the quenching of luminescence of oxygen sensitive dye, and the concentration is proportional to the amount of quenching by oxygen molecules. Importantly, these sensors have excellent resolution at both low ($\pm 0.01\%$ O_2 at 0.21% O_2) and high oxygen concentrations ($\pm 0.1\%$ O_2 at 20.9% O_2) (Presens, 2018).

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For measurement of gaseous oxygen in the sealed vessel, a sensor spot containing oxygen sensitive dye is attached on the inside of the vessel (Figure 6.4A & B). A fibre optic cable is held in place directly opposite and connected to a transmitter, which sends data to a computer to be logged. Dissolved oxygen in the liquid is measured as it passes through a flow through sensor, which is connected at the two ends of the tubing, before the inlet and after the outlet of the liquid channels in the microfluidic device (Figure 6.4C). Oxygen sensitive dye is coated on its inside and an optical fibre connects the sensor to the transmitter.

For validation of the oxygen gradient across the cell channel in the device, COMSOL multiphysics is used to simulate oxygen transport by applying information on the microfluidic geometry and system boundary conditions (see chapter 6.5.8.2). The gradient is experimentally validated (see chapter 6.5.5.3) using a thin film sensor coated with an oxygen sensitive dye and integrated underneath the device (Figure 6.4D). This is to visualise the spatial distribution of oxygen in 2D directly underneath the channels. Oxygen detection is also based on dynamic quenching of luminescence by molecular oxygen.

6.4 Design of the microfluidic device

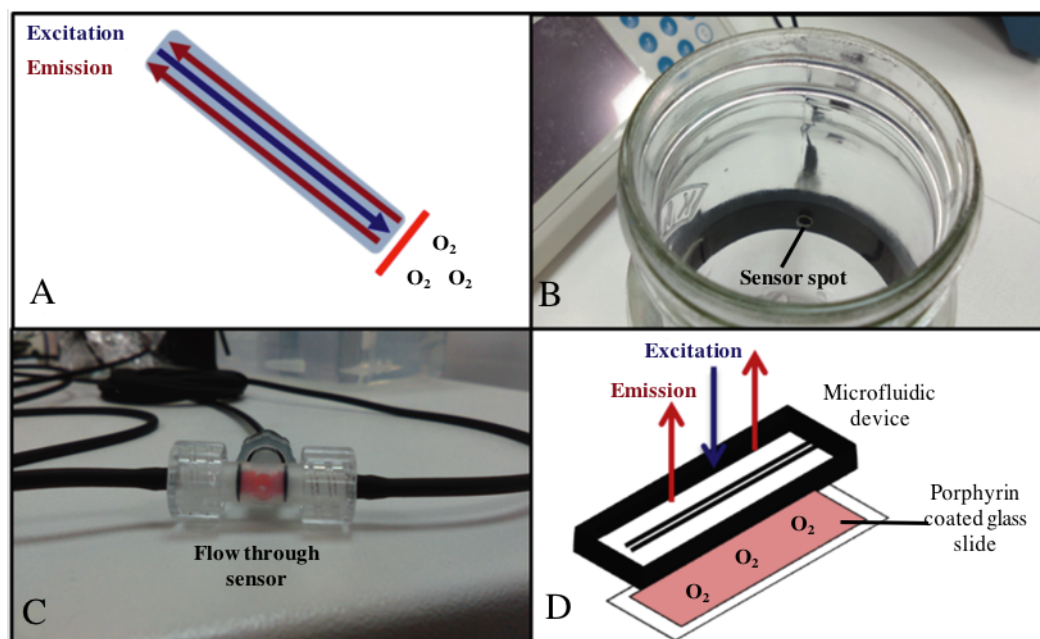


Figure 6.4: **Optical oxygen sensors.** The principle is based on the quenching of luminescence by molecular oxygen. An image of (A) the sensor spot excited by light from a fibre optic cable, (B) the fibre optic cable aligned to the sensor glued to the inside of the glass vessel, (C) flow through sensor and (D) the working principle of the thin film oxygen sensor.

6.4 Design of the microfluidic device

The microfluidic device has been adapted from a previous study investigating EVT migration under chemotaxis (Abbas et al., 2017). The device contains two cell medium channels that are linked via the central cell hydrogel channel. Each of the channels are accessible through two ports and have a length, width and height of 20,300 μm x 1,300 μm and 150 μm respectively. The design is made using AUTOCAD (Figure 6.5A) and the mold fabricated by soft lithography (see material and methods, chapter 3.8.1). The material used for this device is polydimethylsiloxane (PDMS) and is optically transparent, biocompatible and is economical to manufacture (Whitesides, 2006). The length and width of each device is approximately 4.5 cm by 2.3 cm (Figure 6.5B) It is difficult to assess whether

6.4 Design of the microfluidic device

these dimensions are spatially relevant given ethical restrictions over experimental sampling in the first trimester of pregnancy. However, this device has previously been used to find directed EVT migration towards increasing concentrations of the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) in 12 h (Abbas et al., 2017).

The central cell hydrogel channel contains 36 trapezoidal posts on each side in order to confine the gel and allow diffusion of nutrients from medium flowing through the side channels. The number of posts, their spacing and angle was determined experimentally in order to create sufficient surface tension for even gel filling (Farahat et al., 2012). All posts are spaced 100 μm apart and have an angle of 60° (Figure 6.5C). The channel dimensions and flow rates used in this device ensure flow is laminar (Reynolds number range: 0.06 to 20). This makes diffusion and gradient formation in this system more repeatable and reliable.

6.4 Design of the microfluidic device

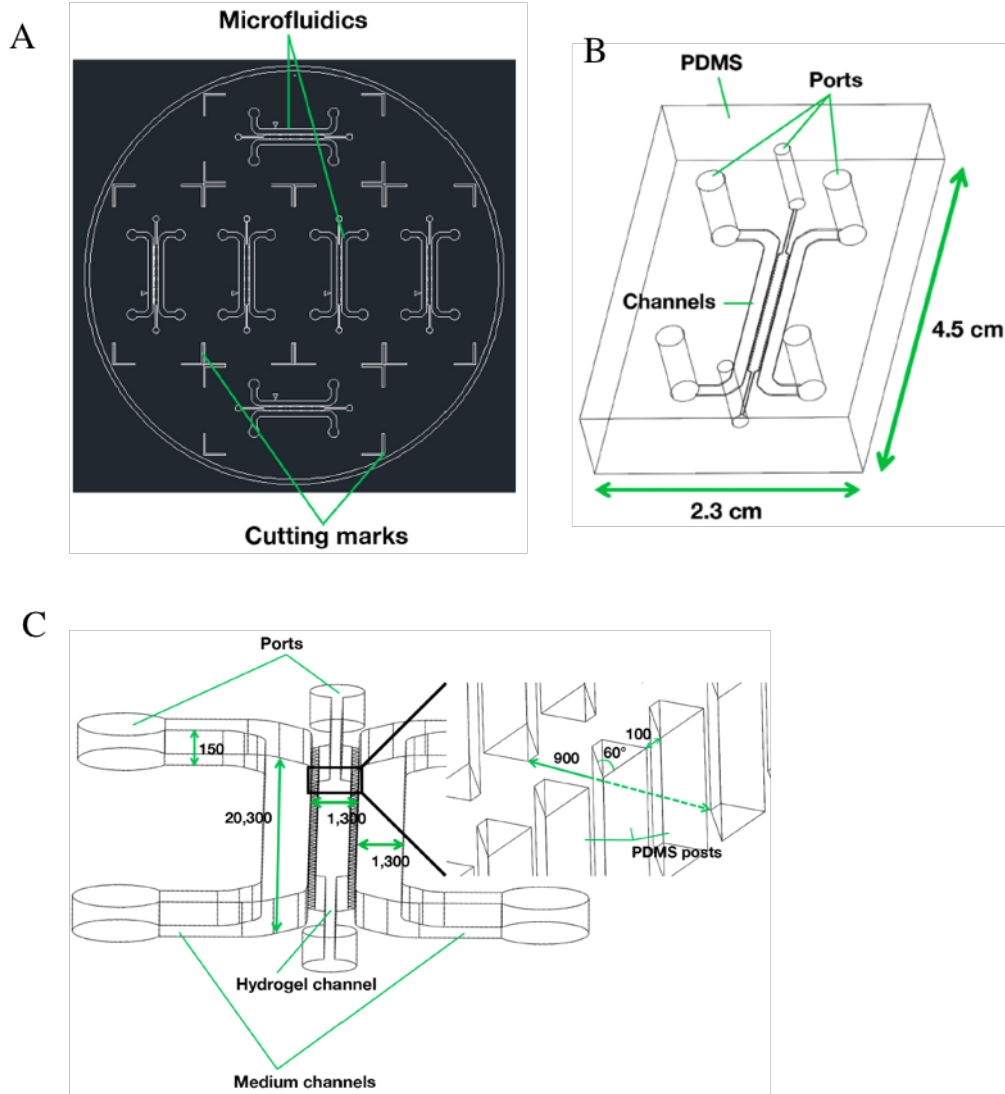


Figure 6.5: **Design of the microfluidic device.** A) The three channel device is designed using AUTOCAD and the mould is fabricated by soft lithography. B) The PDMS device has a width and height of approximately 4.5 cm by 2.3 cm and the (C) length, width and height of each channel is 20,300 μm x 1,300 μm x 150 μm respectively. This image was adapted from (Oefner, 2015).

6.5 Results

6.5.1 Optimising seal of gas vessel

A 500 ml Kilner jar was chosen as the sealed vessel, as it had a large enough volume to contain several meters of tubing. The Kilner jar was designed to be airtight and is economical to purchase ($< \$ 10$). Four holes were drilled (5 cm apart, 1.2 mm drill bit) and hypodermic needles were glued (Araldite, outside diameter: 1 mm) to make ports. Two ports for the inlet and outlet of the liquid, one port for nitrogen gas delivery and one to vent gas. A bubble test was conducted to test for leaks. The vessel was submerged in a water bath with one port connected to a feed of nitrogen gas (< 3 bar or 300 kPa) and the other ports closed by tying a knot of airtight tubing. There was visible release of bubbles from the vessel along the rim of the lid and at the base of the ports. A new lid made from brass metal was cut and a rubber o-ring (internal diameter: 20 cm) was designed to sit between the glass rim and the brass plate. A brass screw cap was used to tighten the seal between the glass rim, rubber o-ring and brass plate (Figure 6.6). High vacuum silicone grease was applied to the glass rim to promote sealing. To stop gas leaking from the base of the ports, the hypodermic needles were soldered onto the brass plate rather than using an adhesive. A bubble test was repeated and there was no visible release of bubbles.

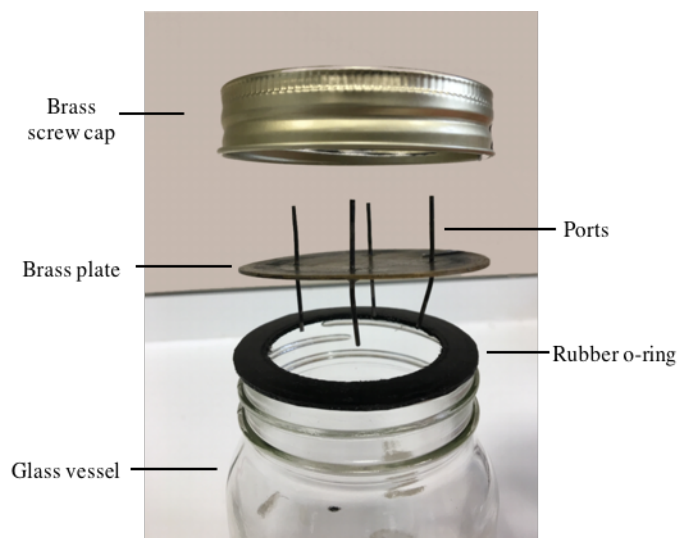


Figure 6.6: **Glass vessel seal mechanism.** High vacuum silicone grease was applied to the rim of the glass and is in contact with a rubber o-ring. The brass plate with ports sits on top of the rubber o-ring and the brass screw cap was used to tighten the seal between the components.

6.5.2 Oxygen concentration was stable for at least 24 hours in the sealed vessel

The oxygen concentration should remain stable in the vessel throughout an experiment. Any leak would alter the driving force of diffusion and disturb the gradient downstream in the device. In addition, a stable oxygen concentration avoids the need for feedback control between the oxygen sensor on the vessel and a nitrogen feed, which would over-complicate the experimental design. Therefore, a more comprehensive leak test was required.

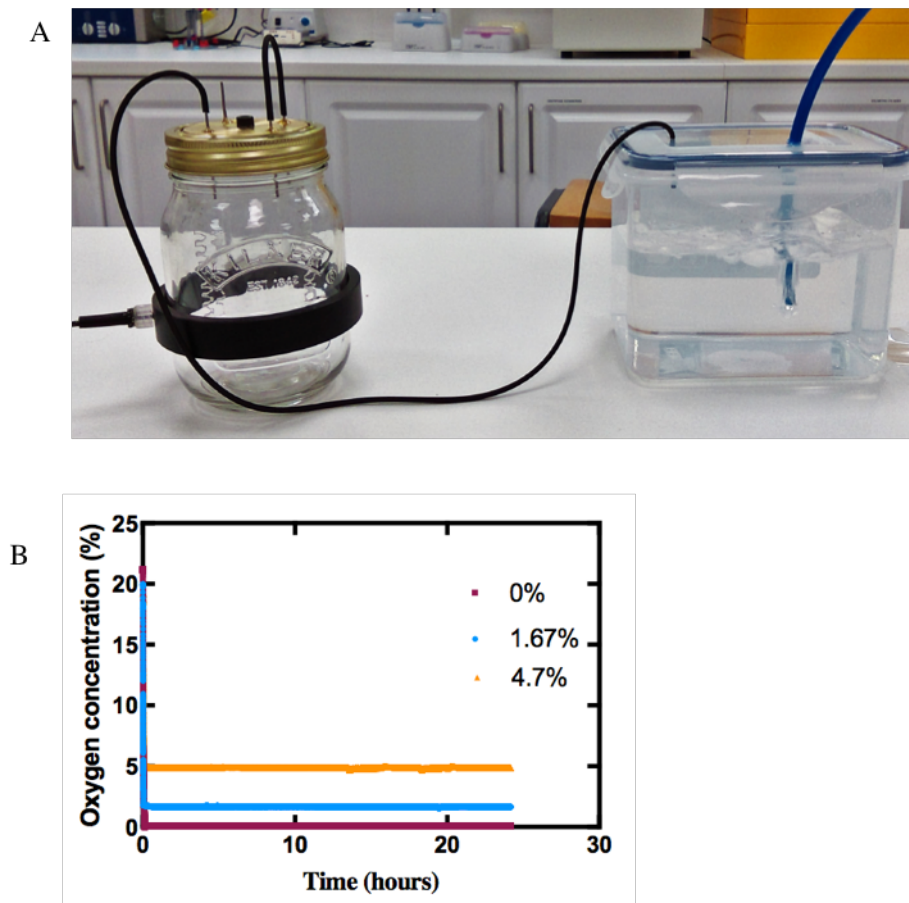


Figure 6.7: **Reducing the oxygen concentration in the vessel.** A) Nitrogen gas was bubbled and fed into the vessel. One port was left open and acts as a vent. The oxygen concentration was measured continuously using an optical oxygen sensor. B) Oxygen was monitored for 24 h at three concentrations between 0-5%, in the region of the lower oxygen concentrations required in the gradient (see Table 6.2).

Nitrogen gas was bubbled and fed to the sealed gas vessel (Figure 6.7A). The oxygen concentration was monitored in real-time with a measurement taken every second. The oxygen concentration was monitored for 24 h at three different concentrations between 0-5% (Figure 6.7B). The change in oxygen concentration from one measurement point to the next was calculated and an average taken for all 86,400 points from time 0 h and when a steady state was reached. Steady

state was reached in approximately 30 min, evidenced by a plateau in the oxygen concentration vs. time curve. The results shown in Table 6.2 show a negligible change in bulk oxygen in the vessel after equilibrium, which validates that the system was leak proof.

$O_2(\%)$	Avg. change in O_2 per second (%)	Avg change in O_2 per second - once steady state is reached (%)	Total change in O_2 - once steady state is reached (%)
0	2.3E-04	1.2E-06	0.1
1.67	2.11E-04	3.6E-07	0.03
4.89	4.5E-05	3.8E-07	0.04

Table 6.2: Sealed vessel leak test.

6.5.3 Simple control of oxygen concentration inside vessel

Real-time monitoring of oxygen inside the vessel allows for control of its concentration. However, as optical sensors are expensive (Witrox 4-channel transmitter, Loligo systems, \$ 8,400) this limits the potential use of this system. Therefore, it was important to determine a relationship between oxygen concentration inside the vessel and a variable that is easily controlled. In this system, a nitrogen tank was used and nitrogen was bubbled at a fixed flow rate, 12.5 l/min, in a fixed volume of water, 500 ml. A time dependent relationship between the oxygen concentration inside the vessel and duration of nitrogen bubbling was determined (Figure 6.8). See material and methods, 3.9.3 for details.

6.5.4 Diffusion of gas mixture

Diffusion of dissolved oxygen flowing through the polymer tubing and into the sealed vessel is governed by Ficks law of diffusion. Beyond this theoretical description, it is important to determine parameters that can be adjusted, which control rate and amount of oxygen diffusion experimentally. This is to enable

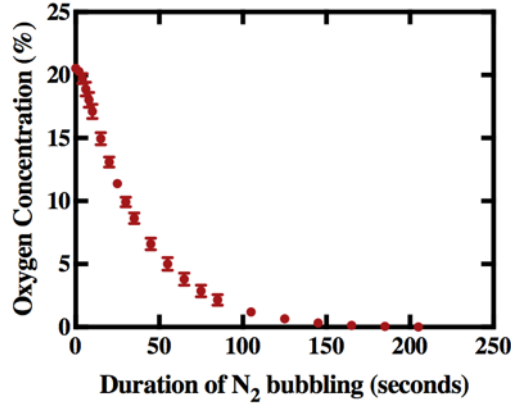


Figure 6.8: **Time dependent relationship between oxygen concentration in the vessel and duration of nitrogen (N₂) bubbling.** The oxygen concentration in the vessel can be controlled without the need for optical oxygen sensors, which are expensive to procure. Nitrogen is bubbled at a fixed flow rate, 12.5 l/min, in a fixed volume of water, 500 ml. Mean $\pm$ SD. N= 4.

control of the gradient downstream in the device. In this study, polymer tubing with the highest gas permeability available on the market and with the smallest membrane thickness was chosen. This parameter is fixed and is a starting point to allow for rapid diffusion. The concentration gradient of oxygen and flow rate of liquid through the system were investigated as parameters for oxygen control.

6.5.4.1 Driving force - concentration gradient

The force driving oxygen molecules through the polymer membrane is the concentration gradient. Oxygen diffusion along the steepest gradient, from 21% dissolved in the liquid to 0% in the vessel was investigated. The concentration of oxygen in two vessels, one for each medium side channels were reduced to 0% and then placed in an incubator at 37 °C, to reflect conditions in a cell experiment. Because the oxygen transmitter is limited to four channels and one is required to monitor oxygen in the vessel, only dissolved oxygen at the inlet and outlet of one side channel was monitored.

The reduction of dissolved oxygen to below 1% recorded at the inlet of the device takes 4 ± 2 min (n=3) (Figure 6.9A). The oxygen concentration in the

vessel and at the inlet and outlet of the device was stable for 10 h (Figure 6.9B). Linear regression analysis on the slopes show there was no significant deviation from zero ($p > 0.05$). The average difference between the vessel and device inlet concentration was $0.01 \pm 0.1\%$ ($n=3$) and there was no significant difference between their slopes. This demonstrates the diffusion capabilities in the vessel.

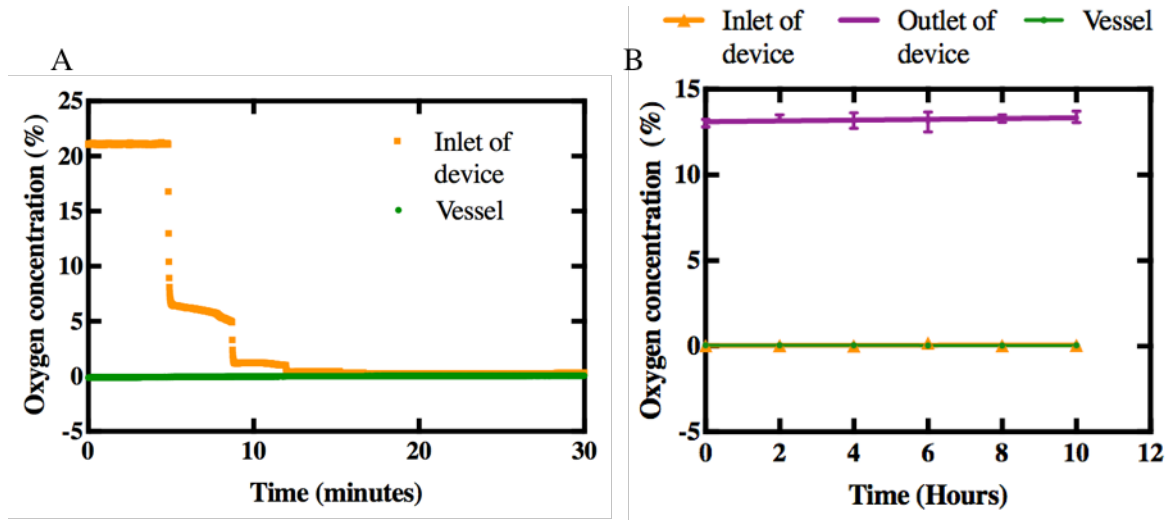


Figure 6.9: **Driving force for diffusion - concentration gradient.** The oxygen concentration in the vessel was reduced to 0% and deionized water was perfused through the vessel in gas permeable tubing. (A) It takes 4 ± 2 minutes for the dissolved oxygen measured at the device inlet to reduce from 21% to $<1\%$. (B) The oxygen concentration in the vessel and at the inlet and outlet of device was stable for 10 h. Mean $\pm$ SD. $N=3$.

The high gas permeability of PDMS allows for rapid diffusion of oxygen between channels, which makes it well suited to generate gradients. However, it was found to result in oxygen increasing at the outlet of the device to $13.2 \pm 0.33\%$ ($n=3$). This was hypothesised to be the result of oxygen diffusion from atmosphere. This increase means an oxygen gradient across the central gel containing channel, would not be uniform along the length of the device.

6.5.4.2 Optimising oxygen permeability of the microfluidic device

Several methods were attempted to reduce oxygen diffusion into the device from atmosphere. First, the device was plasma treated, which is known to reduce the diffusion coefficient of oxygen in PDMS (Markov et al., 2014). Devices are converted from hydrophobic to hydrophilic, which removes surface methyl groups and replaces them with hydroxyl groups making the exposed surface to resemble silica, a gas impermeable substance. Plasma treatment for 5 minutes at 70 W, is known to render PDMS hydrophilic for 6 hours (Markov et al., 2014). Separate devices were plasma treated for a duration of 1, 2, 3 and 5 minutes and the dissolved oxygen concentration was monitored one hour after at the inlet and outlet of the device as described in 6.5.4.1. There was no change in the outlet oxygen concentration ($13.0 \pm 0.2\%$, unpaired t-test, $P > 0.5$, $n=4$) after perfusion of deoxygenated water for two hours. A list of optimising steps taken in the development of a microfluidic oxygen gradient system is shown in Table 6.3.

Next a layer of plastic (polyvinylidene chloride) film was coated onto the top and sides of the device to act as an impermeable barrier to oxygen. The film was coated by applying a thin layer of vacuum grease onto the pdms surface. After coating, a biopsy punch was used to make a hole at the insert of the ports. However, there was no change in the oxygen concentration ($13.1 \pm 0.3\%$, unpaired t-test, $P > 0.5$, $n=3$) in the outlet of the device after perfusion of deoxygenated water for 2 hours. It is hypothesised that oxygen stored in the PDMS is readily diffusing into the two side channels as deoxygenated liquid is perfused.

6.5.4.3 Driving force - flow rate

Oxygen at the outlet of the device decreases as flow rate increases as there was less time for oxygen diffusion from atmosphere (Figure 6.10A). At 54 ml/hr the difference in oxygen recorded at the inlet and outlet was 1.15% and parity is expected as flow rate tends to infinity. However, most syringe pumps are limited in syringe capacity, typically between 60-100 ml. At high flow rates, syringes would need to be changed leading to air bubble generation causing irregularities in gradient formation. The experiment was repeated for flow rates 6-54 ml/hr using a peristaltic pump, which circulates liquid continuously avoiding the need

	Problem	Optimising steps	Experimental assessment
Seal of gas vessel	Gas leak at rim of vessel and at the base of ports	Hyperdmic needles were soldered rather than glued onto plate A rubber o-ring was placed between vessel and brass plate	A bubble leak test allows visible identification of the leak
Gas-liquid diffusion system	Increasing rate of diffusion inside the vessel	The length of tubing was increased from 1.5 to 10 m Wall thickness decreased from 1 to 0.5 mm	The oxygen concentration was measured at the inlet and outlet of the gas permeable tubing after oxygen from the vessel was depleted. The time it took for oxygen to reduce from 21% to <1% at the outlet of the vessel reduced from 12 to 4 min
Microfluidic device permeability	Reduce permeability of the device to oxygen	Plasma treat for 1-5 min to render surface hydrophobic Coat with gas impermeable film, polyvinylidene chloride	There was no change in the oxygen concentration at the outlet of the device, with an average of 13.0% (plasma treat) and 13.1% (plastic film) after deoxygenated liquid was perfused for 2 h.
Gel loading in the microfluidic device	Detachment of the gel from the posts in the central channel	Increase stiffness of Matrigel by making a 50:50 Matrigel-collagen composite	There was detachment of the gel with flow rates above 6 ml/hr
	Detachment of the gel from the posts in the central channel	Alter surface chemistry: add Poly-D-Lysine and incubate device at 80 °C for between 48 and 72 h before gel filling to render channels hydrophobic	There was detachment of the gel with flow rates above 6 ml/hr

Table 6.3: Optimising steps taken in the development of a microfluidic oxygen generation system.

6.5 Results

for syringe changes. There was no significant difference (unpaired t-test, $n=3$, $p > 0.05$) in outlet oxygen concentration between the syringe and peristaltic pump (Figure 6.10B).

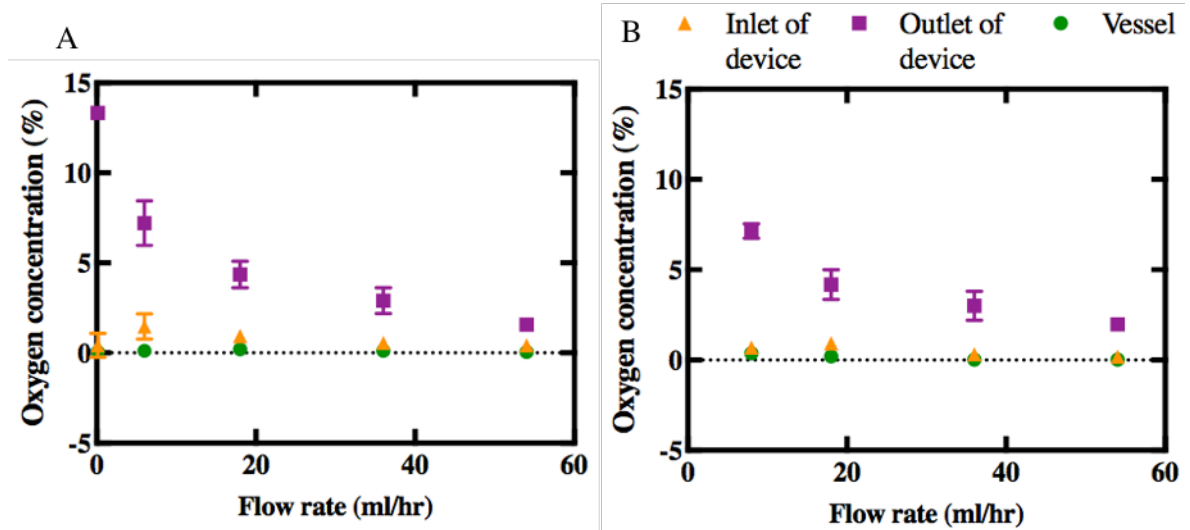


Figure 6.10: **Oxygen concentration as a function of flow rate.** Oxygen at the outlet of the device decreases as flow rate increases for flow generated using (A) a syringe pump and (B) a peristaltic pump, which circulates the initial volume in the system continuously. Mean $\pm$ SD. $N=3$.

6.5.5 Visual validation of gradient

In recent years, advancements have been made in the integration of sensors into bioreactors and microfluidic devices. Commercial systems range from on point measurements using microparticles to fluorescent sensor foils, which are integrated into devices and provide a 2D readout. Both these systems use oxygen sensitive luminescence quenching systems based on established collisional luminescence quenching scheme. In this work, a thin film oxygen sensor was fabricated and integrated into the microfluidic device to provide a visual read out of oxygen at the base of the channels. Fluorescent light or a confocal microscope can be used to excite the oxygen sensitive dye and the emission is proportional to the oxygen concentration.

6.5.5.1 Optimising the thin film sensor

The protocol is based on work by Ungerbock *et al.*, who use an oxygen sensitive dye, platinum(II)-5,10,15,20-tetrakis-(2,3,4,5,6- pentafluorophenyl)-porphyrin (Pt-TFPP) (Ungerböck *et al.*, 2013). Full details of sensor fabrication are described in materials and methods, chapter 3.9.2.2. The thin film sensor was applied using a bar film applicator (Figure 6.11A) and imaged at an excitation wavelength of 405 nm. The emission intensity at 650 nm was proportional to the oxygen concentration (Figure 6.11B). The interface between the thin film sensor and the glass slide at atmospheric conditions, is shown in Figure 6.11C.

There were challenges in producing a uniform layer of sensor film, with streaks visible when imaged at atmospheric conditions (Figure 6.11D). It was hypothesised that this was caused by agglomeration of particles that get trapped in the lip opening of the bar film applicator leading to flow disturbances. Filtration and particle sonification did not reduce the effect of streaks.

Alternatively, spin coating was used to apply the film (200 μ l at 2500 rpm for 5 min). However, there were inconsistencies in the distribution of sensor solution, hypothesised to arise from the high vapour pressure of chloroform (Pubchem, 2017a). Toluene was tested as its rate of evaporation is lower, (Pubchem, 2017b) which resulted in a homogenous coating and there were no streaks visible when imaged under the microscope. The sensor film is shown in Figure 6.11E. The thickness of the film was determined using a Taylor Hobson surface profiler, as described in materials and methods, chapter 3.9.2.2. The thickness was found to be $5.23 \pm 0.86 \mu\text{m}$ (n=3).

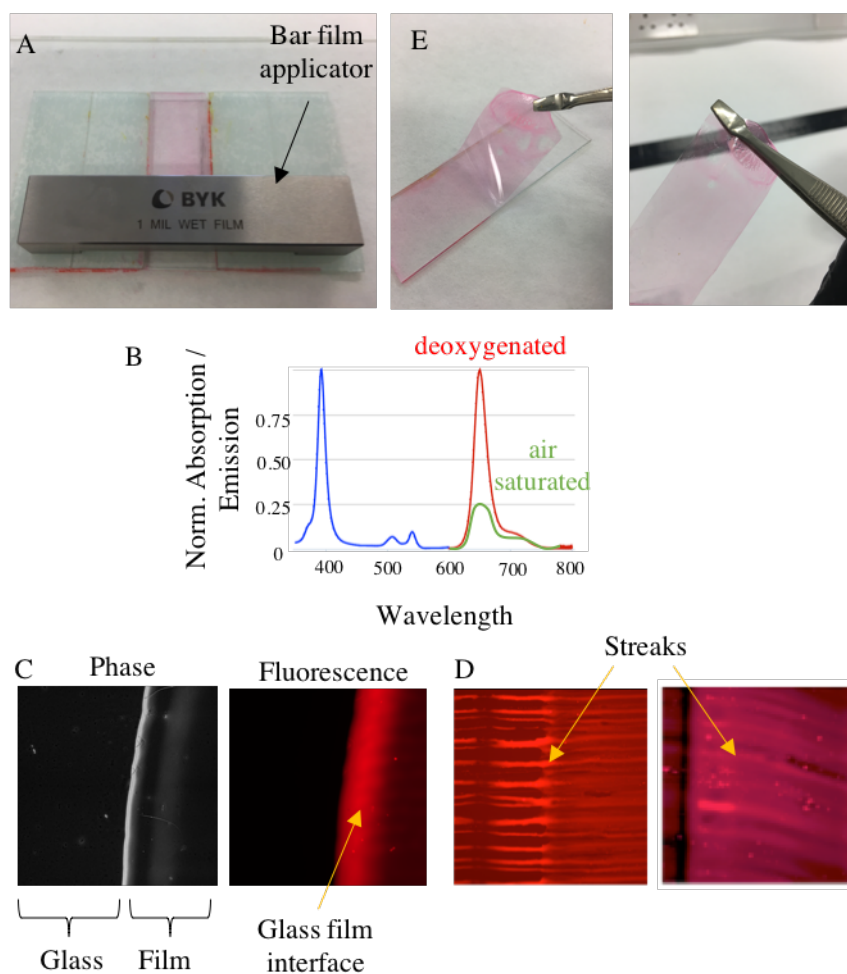


Figure 6.11: **Sensor film optimisation.** A) Sensor solution made up of chloroform, polystyrene and oxygen sensitive dye PtTFPP was pipetted onto a glass slide and was knife coated using a bar film applicator. B) The sensor was excited at 405 nm and its emission is a function of oxygen concentration. C) There is a clear interface between the glass and thin film sensor in both phase and fluorescence imaging. D) Knife coating the sensor solution lead to visible streaks and resulted in a change in the method of application, to spin coating. The sensor film, after being peeled off from the glass slide is shown in (E).

6.5.5.2 Testing the thin film sensor

Devices with an integrated thin film sensor were kept away from light and used no more than one week after fabrication (6.12A). To test if the sensor emission changes with oxygen concentration, nitrogen gas was bubbled through the vessel with gas impermeable tubing connecting the vessel to the inlet of the central channel of the device. An image was taken every 30 seconds close to the inlet of the central gel channel. The concentration in the vessel was monitored in real-time using the oxygen sensitive spot and witrox oxygen transmitter.

Once reduced to 0% in the vessel, the gas feed to the device was switched off and the tubing connecting the channel was disconnected, allowing oxygen in the channels to return to atmospheric levels. The emission of the PtTFPP dye was found to increase with decreasing oxygen concentrations (Figure 6.12B). This is in accordance with the quenching principle of the dye and the relationship between emission intensity and dye quenching is given by the Stern-Volmer equation:

$$\frac{I_0}{I} = 1 + K_{SV} + 1 \quad (6.2)$$

where, I_0 is the intensity or lifetime of luminescence without the quenching molecule (pure N_2), I is the intensity or lifetime of luminescence with the quenching molecule (O_2), K_{SV} is the Stern-Volmer constant. An average intensity of each image was quantified using FIJI and a plot of I_0/I vs oxygen concentration for three experiments is shown in Figure 6.12C. The plot shows a linear relationship ($R^2 = 0.9791-0.9941$), obeying the equation in (4). From the slope, $K_{SV} = 5.9 \times 10^{-2} \pm 3.3 \times 10^{-3}$ (% O_2).

Finally, liquid was perfused at 10 ml/hr through the sealed vessel filled with nitrogen and into the central channel of the device, to determine whether the sensor can detect deoxygenated liquid. Detection is shown in Figure 6.12D, with liquid in the central channel, which has leaked into one of the side channels. There was limited signal from the sensor in the channel with no liquid and from an air bubble in the middle of the central channel.

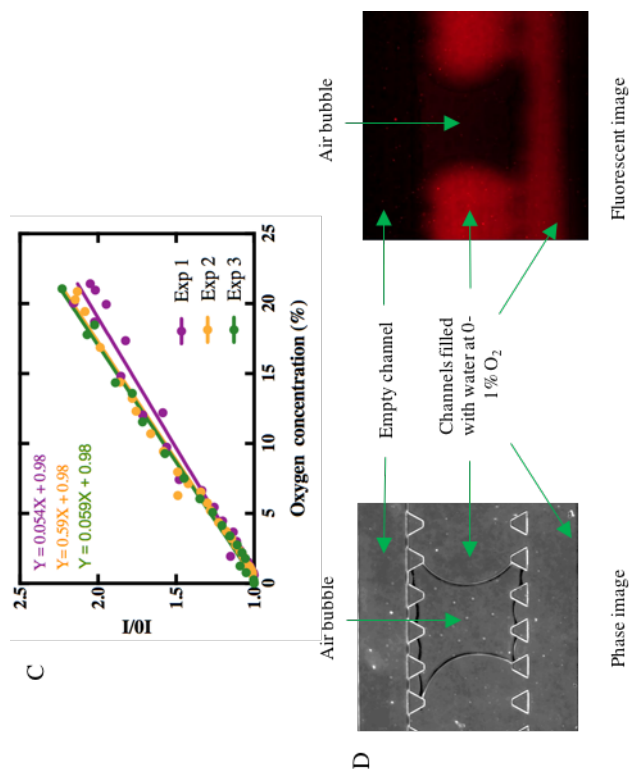


Figure 6.12: **Testing the thin film oxygen sensor.** A) A layer of PDMS was spin coated on top of the sensor film, which facilitates bonding to the microfluidic device. B) To test the responsiveness of the sensor to low oxygen, N₂ gas was bubbled and fed into the sealed vessel and then into the inlet of the central channel. The light emitted by the oxygen sensitive dye increases as oxygen decreases. C) An average intensity of the image was determined using FIJI and all values were normalised showing a linear relationship between image intensity and oxygen. D) Low oxygen liquid equilibrated in the sealed vessel was perfused through the central channel, which then leaked into one of the two side channels. This allowed confirmation of sensor signal to low oxygen liquid in the two channels that were filled. As expected, there was limited signal where there is an air bubble in the central channel.

6.5.5.3 Oxygen gradient validation

To test if an oxygen gradient can be detected visually using the thin film sensor, deionized water equilibrated to 0% oxygen was perfused into the side channels of a microfluidic device. Two gas sealed vessels were used for two flow rates, at 0.1 ml/hr and 10 ml/hr for each side channel. Agarose gel (0.5% w/v) was embedded in the central channel because it is able to withstand higher flow rates than Matrigel and it is inexpensive. Liquid was allowed to perfuse through the vessels and the device for 10 h before the microscope was switched on. The centre of the device was imaged using a 405 nm laser to excite the PtTFPP dye (Figure 6.13A) with a corresponding phase image taken (Figure 6.13B). For the control experiment, deionized water enters the device at atmospheric oxygen at the same flow rates as the gradient experiment (Figure 6.13C). Three cut lines were taken across the central channel at different positions and the normalised intensity values (division by the largest intensity value) determined using FIJI (Figure 6.13D). Oxygen decreases across the width of the channel, with linear regression analysis giving a R^2 of 0.91, 0.88 and 0.86 for the three lines compared to R^2 of 0.21 for the control. The slope of the average of the lines were significantly different to that of the control ($p < 0.0001$).

It was shown in Figure 6.9, that oxygen increases along the device and this was hypothesised to be a result of oxygen entering from atmosphere. The normalised intensity of three cut lines along the central channel were determined to assess change in oxygen along the length scale of the image (Figure 6.13E). A linear regression analysis was performed with R^2 of 0.2, 0.3 and 0.31 compared to R^2 of 0.38 for the control. There was no significant difference between the slope of the control and the average of the three cut lines ($p > 0.5$). This shows that changes in oxygen along the device are not having an effect on measured oxygen intensity at the length scale of an image ($y=2.8$ mm). This is the field of view of the objective lens that would be used to track the cells.

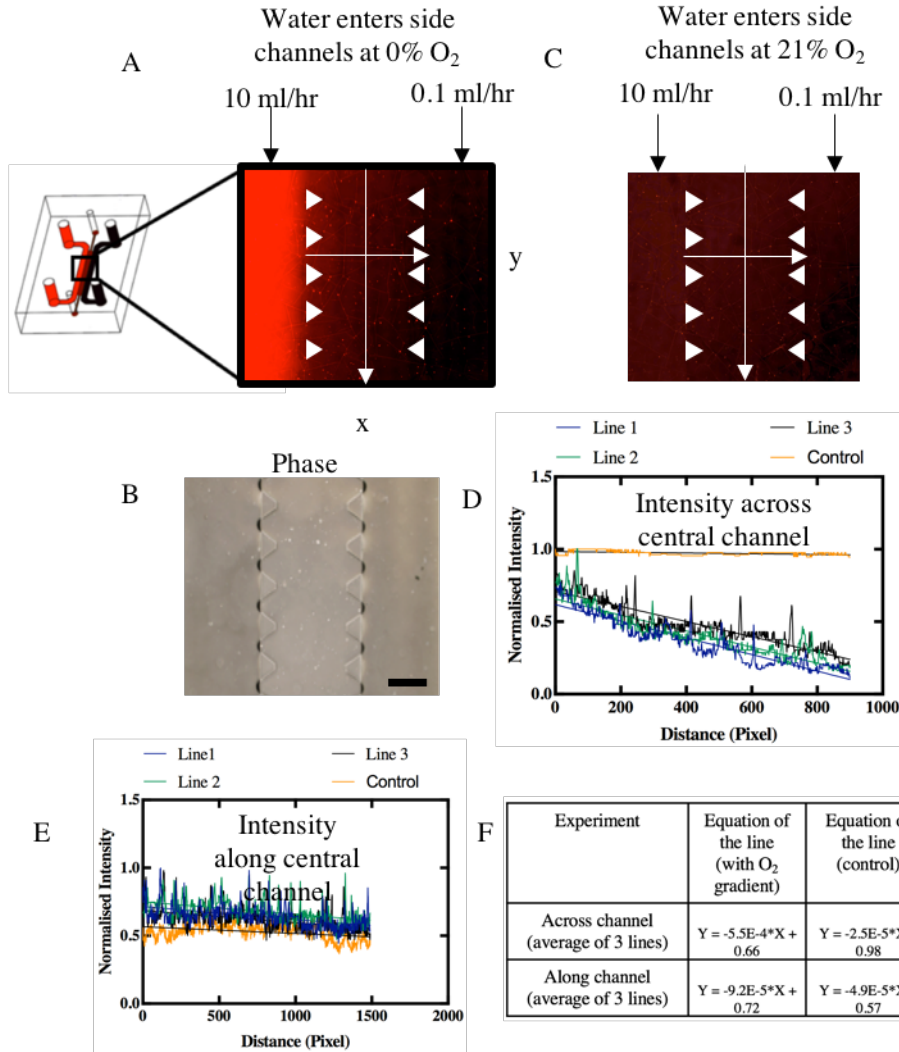


Figure 6.13: **Confirmation of an oxygen gradient across the device.** A) Liquid equilibrated to 0% oxygen was perfused through two side channels, at 10 ml/hr and 0.1 ml/hr. The thin film sensor was excited at 405 nm and images of the centre of each device were acquired, (B) as well as in phase. C) For the control, liquid was perfused at atmospheric oxygen. D) The normalised intensity is plotted for three cut lines, which shows decreasing levels of oxygen across the central channel. E) The oxygen profile along the channel was determined and the normalised intensity for three cut lines shown. For both controls, the line shown is an average of three measurements. F) The equation of the lines determined by regression analysis for oxygen measured across and along the central channel. Scale bar = 500 μ m.

6.5.6 Gel loading in the microfluidic device

Now that flow rate has been established as a parameter to control oxygen in the device, it is important to determine the limits to what the device can handle. Cells will be encapsulated in Matrigel, a basement membrane used for migration studies. From results in chapter 4, Matrigel is a soft material (331.6 ± 105 Pa) and its stiffness is positively correlated with concentration (dilution in cell medium). Due to its soft nature, Matrigel was found to detach easily from the central channel when liquid was pipetted into the side channels. Because of this, it was necessary to ask if there is risk of detachment at flow rates used in this study.

Three Matrigel concentrations, first diluted to 98% v/v with the addition of phenol red for visualisation and to a further 78% v/v and 48% v/v with phosphate-buffered saline (PBS), were tested. These solutions were pipetted into the central channel of the device and allowed to crosslink at 37 °C for 30 min. A syringe pump was used to generate flow of deionized water between 0.12 ml/hr and 54 ml/hr. The experiment was carried out under an EVOS lab bench microscope at room temperature for one hour. There was detachment in all concentrations of Matrigel from the channel posts for flow rates including and above 6 ml/hr (Figure 6.14). Matrigel detachment occurred when repeated under an environmental chamber at 37 °C using a confocal LSM microscope. Collagen I was added to Matrigel to make a 50:50 composite, which increases material stiffness (Dewitt et al., 2009), but this had no effect on gel detachment.

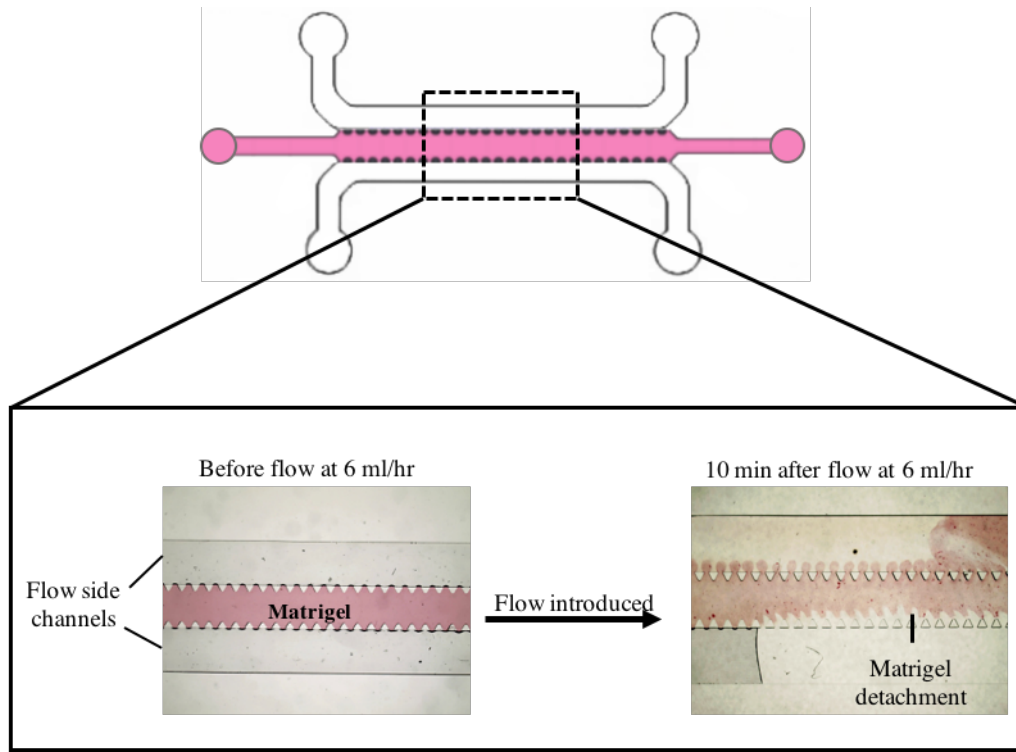


Figure 6.14: **Matrigel detachment in the central gel channel.** Matrigel is confined by 36 trapezoidal posts in the central channel of the device. When flow is introduced in the two side channels, the gel detaches from the posts at flow rates greater than and including 6 ml/hr.

6.5.7 Altering surface chemistry

In microfluidics, surface effects play a dominant role as the surface-to-volume ratio increases, which may have an effect on gel attachment onto the posts in the central channel. To investigate if altering surface chemistry can improve gel attachment, 1 mg/ml of Poly-D-Lysine (PDL) solution was pipetted into all channels within 20 min of plasma bonding the device to glass, and incubated for 6 h at 37 °C (Farahat et al., 2012). PDL is positively charged and it is hypothesised that PDL treated channels might enhance adhesion to Matrigel, as basement membranes are negatively charged (Miner, 2012). However, addition of PDL did not improve Matrigel attachment, with detachment observed within 1 h at all flow rates and gel concentrations. An interface that is robust to spillage

is one that can withstand large pressure perturbations. The pressure differential sustained by surface tension ΔP is given by:

$$\Delta P = \gamma \left(\frac{1}{R_x} + \frac{1}{R_z} \right) \quad (6.3)$$

where, γ is the surface tension coefficient, and R_x and R_z are the radii of curvature of the air liquid interface in two mutually perpendicular directions. Farahat *et al.*, used this relationship to optimise trapezoidal post spacing in the original design of the device, to ensure the device could withstand filling pressures during filling of the gel solution (Farahat *et al.*, 2012).

To maximize ΔP , the radii of curvatures need to be minimized. R_z is determined by the channel height and R_x is governed by post spacing, which are both fixed geometries. The hydrophobicity of the air-liquid and gel-liquid interface, will also affect R_x . The hydrophobicity of PDMS was optimised to investigate if this could result in a smaller R_x , in line with findings by Farahat *et al.* PDMS is inherently hydrophobic but becomes hydrophilic after plasma treatment when used for bonding to glass (Bodas and Khan-Malek, 2007).

During Matrigel filling the gel bulges between the PDMS posts, resulting in a large R_x but the gel solution-air interface curvature can sustain filling pressures (Figure 6.15A). But after incubation at 37 °C for Matrigel cross-linking, the gel-air interface was concave, with air pockets remaining even after the side channels were filled with liquid. The gel-liquid interface appears hydrophilic, with a large radius of curvature, which makes the gel prone to detachment (Figure 6.12B). To render PDMS hydrophobic, the device was incubated at 80 °C for between 48 and 72 h before gel filling. This resulted in a flattening of Matrigel at the interface (Figure 6.15C) but there was no change when flow was introduced at 6 ml/hr with gel detachment within one hour. The experiment was repeated at flow rates between 0.12 ml/hr and 0.6 ml/hr to determine the threshold of the system. At the same Matrigel concentrations described in Chapter 6.5.6, flow at 0.24, 0.36 and 0.6 ml/hr was introduced and detachment was observed at 0.6 ml/hr within one hour. Thus, altering the surface chemistry had no effect on preventing gel detachment, and the geometry based on gel confinement within trapezoidal posts

was not cohesive to flow rates greater than 0.36 ml/hr. Given the need to operate at flow rates larger than this, the device was re-designed.

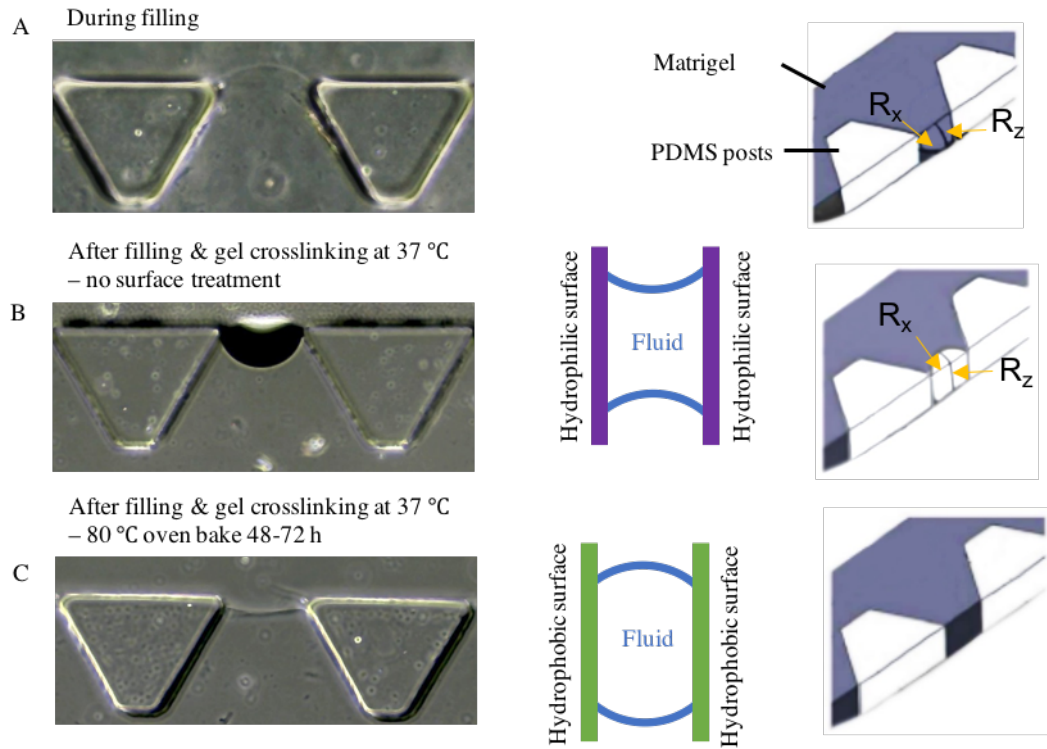


Figure 6.15: **Altering surface chemistry of PDMS.** A) During filling, the gel can withstand filling pressures and stays confined within the 36 trapezoidal posts. (B) After Matrigel cross-linking at 37 °C, the gel was concave, hydrophilic in nature and prone to detachment from the PDMS posts. To increase the pressure differential sustained by surface tension, the radii of curvature needs to be minimised. PDMS was rendered hydrophobic by baking the device for between 48-72 h before gel filling. (C) The gel is now flat at the gel-air interface, however, this did not result in an improvement in the tolerance of the gel at flow rates greater than and including 6 ml/hr. Image adapted from (Farahat et al., 2012).

6.5.8 Microfluidic device re-design

The device was re-designed, replacing the trapezoidal posts with a closed channel to confine the gel. The central channel is now separated by PDMS, with pre-

equilibrated liquid flowing in channels either side to generate an oxygen gradient. As PDMS is gas permeable, the pre-equilibrated liquid acts as a sink to oxygen in the central gel channel. All channel dimensions are the same as the trapezoidal post design described in chapter 6.4. Before sending the design for wafer manufacture, COMSOL multiphysics was used to conduct a parametric study on the separation distance between the side channels and the central gel channel. The distance needs to be small enough for rapid oxygen diffusion but within the range for reliable soft-lithography manufacture.

Due to time pressure, validation experiments could not be repeated. It is assumed that the results in the trapezoidal device can be correlated to the redesign for the following reasons. First, the dimensions of each channel are the same for both devices. Second, the 36 trapezoidal posts on each side are made from PDMS with Matrigel or agarose (used to model Matrigel) filling in the 100 μm gap. The experimentally determined diffusion coefficient of oxygen in Matrigel ($1.5 \times 10^{-9} \text{m}^2/\text{s}$ at 37°C) and 5% (w/v) agarose ($1.99 \times 10^{-9} \text{m}^2/\text{s}$ at 30°C) is at the same order of magnitude as PDMS ($3.21 \times 10^{-9} \text{m}^2/\text{s}$ at 37°C) (Colom et al., 2014; Hulst et al., 1989; Markov et al., 2014).

6.5.8.1 COMSOL channel separation study

COMSOL multiphysics (version 5.3) was used to simulate the oxygen gradient across the central gel channel of the device. COMSOL is comprised of different modules tailored to different applications in electrical, mechanical, fluid and chemical engineering. To simulate the gradient across the gel channel, the ‘transport of diluted species’ module was used. It solves for the velocity and pressure of the flow, as well as the concentration of the diluted species, which is oxygen in this model. The geometry, material properties, model boundary conditions, fluid and diluted species initial conditions all need to be defined before the model is run.

To determine optimal channel separation distance, a simplified geometry was built using COMSOL’s own geometry builder. This makes it easy to conduct a parametric study at separation distances between 20 μm and 120 μm , rather than importing each geometry from the design software SOLIDWORKS. Three

channels with channel height, length, width as well as inlet and outlet area were kept the same as the trapezoidal based device. The channels were placed in the centre of a box to model the PDMS device and each channel and channel port were defined as separate geometries. This is to ensure all boundaries are well defined. A no flux boundary condition was set to represent the glass slide bonded to the PDMS.

The concentration of oxygen dissolved in water entering the device was 0 mol m^{-3} with no concentration set for oxygen at the outlet. The PDMS box, the gel in the central channel and channel boundaries were set to 21%, with temperature at 37°C . The diffusion coefficient for PDMS, water and Matrigel were defined as listed in 6.5.8. The range of flow rates experimentally tested (in chapter 6.5.4.2) of liquid entering the two side channels (between 0.1 ml/hr and 60 ml/hr) were modelled. To enable solving of the partial differential equations that govern the model, the flow domains were split into smaller subdomains made up of tetrahedra in 3D and are called elements. There were 167,208 elements with a maximum and minimum element size of 1.51 mm and 0.06 mm respectively.

Once the model was solved, a cut through line was drawn across and along the centre of the gel channel (Figure 6.16A). A separation distance of $100 \mu\text{m}$ was found to be the limit for oxygen gradient generation across the central channel for flow rates between 0.1 ml/hr and 60 ml/hr . The concentration profile at flow rates of 0.1 ml/hr and 10 ml/hr through inlet A and inlet B respectively are shown in Figure 6.16B and plotted in Figure 6.16C. These flow rates were found to produce a gradient that approaches physiology at the maternal-fetal interface (Jauniaux et al., 2000). They were inputted back into this geometry to confirm gradient generation. A separation distance of $80 \mu\text{m}$ was chosen for the design. Although the minimum feature size of conventional lithography is $1\text{-}2 \mu\text{m}$ in lateral dimension (Qin et al., 2010), a separation distance much larger than this was chosen. It was hypothesised that the surface area needed for bonding to generate a seal between the channels requires a greater surface area than provided at the minimum dimension. In addition, small distances between resin on the mold used to imprint the channels are more likely to come off after continuous PDMS peeling. There were also concerns that the gel may be disturbed by the flow of liquid in the side channels at small separation distances.

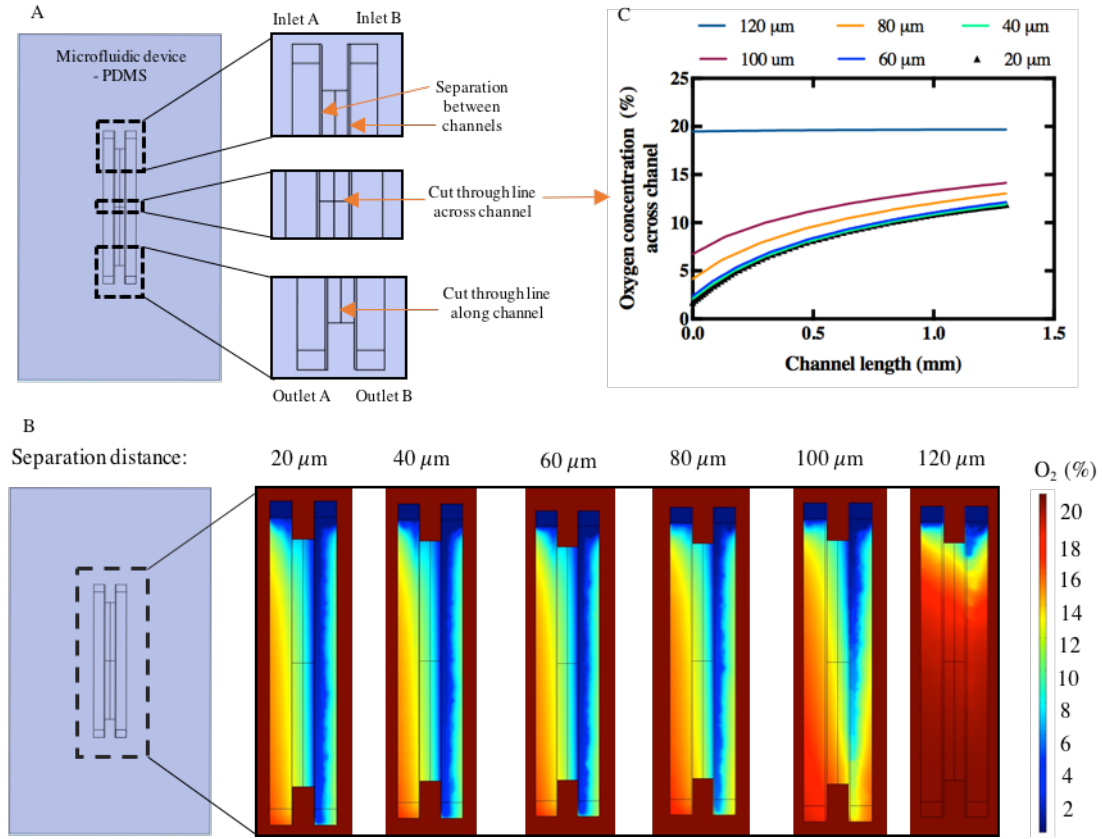


Figure 6.16: **Channel separation parametric study.** (A) Oxygen profiles in three channel microfluidic device modeled in COMSOL. Water enters the two side channels via inlet A and inlet B at 0.1 ml/hr and 10 ml/hr respectively. (B) Oxygen concentration profiles at channel separation distances between 20 μm and 120 μm . (C) Oxygen concentration along the cut through line across the central gel channel.

6.5.8.2 COMSOL oxygen gradient validation

To validate the oxygen gradient, it was important to do so in a geometry that is comparable to what will be manufactured. The layout was first created in AutoCAD in 2D (Figure 6.17A) and exported to SOLIDWORKS to generate a 3D drawing. AutoCAD and SOLIDWORKS are computer aided design software that aid in the creation and optimisation of 2D and 3D drawings. All boundaries were defined in SOLIDWORKS before exporting the geometry to COMSOL

(Figure 6.17B). The model parameters were identical to the simulation described in the channel separation study in chapter 6.5.8.1. The number of mesh elements generated was 189,824 with the maximum and minimum element size, 2.4 mm and 0.18 mm respectively.

Generation of the tetrahedral mesh is illustrated in Figure 6.17C. The model was run for a range of flow rates between 0.12 ml/hr and 54 ml/hr with selected concentration profiles shown in Figure 6.17D. At 0.12 ml/hr and 0.36 ml/hr in both inlet channels there is an oxygen gradient along the central channel, but not across. To generate a gradient across the channel, the flow rate in one of the inlets needs to be higher than the other. This is illustrated when the flow rates are both close (0.12 ml/hr and 0.6 ml/hr) in range and far apart (0.12 ml/hr and 18 ml/hr). A parametric study was conducted at different combinations of flow rates at inlet A and inlet B. The oxygen profile at flow rates 0.1 ml/hr and 10 ml/hr is shown in Figure 6.18A. The gradient measured across the centre of the channel was from 2.92% to 10.1%, which meets the study requirements (1-3% to 8-10%) (Figure 6.18B). Non-linear regression analysis was performed and a second order quadratic equation was fitted, with $R^2 = 0.99$. The oxygen profile along the full length of the central channel (Figure 6.18C) and along the length scale of an image taken by a 10 x objective lens (Figure 6.18D) was estimated. To compare the oxygen profiles, the change in oxygen concentration (ΔO_2) over distance was estimated. This was found to be 5.4, 0.08 and 0.08 $\Delta O_2(\%) / \text{mm}$ across the central channel, along the full length of the central channel and along the length scale of an image taken by a 10 x objective lens respectively.

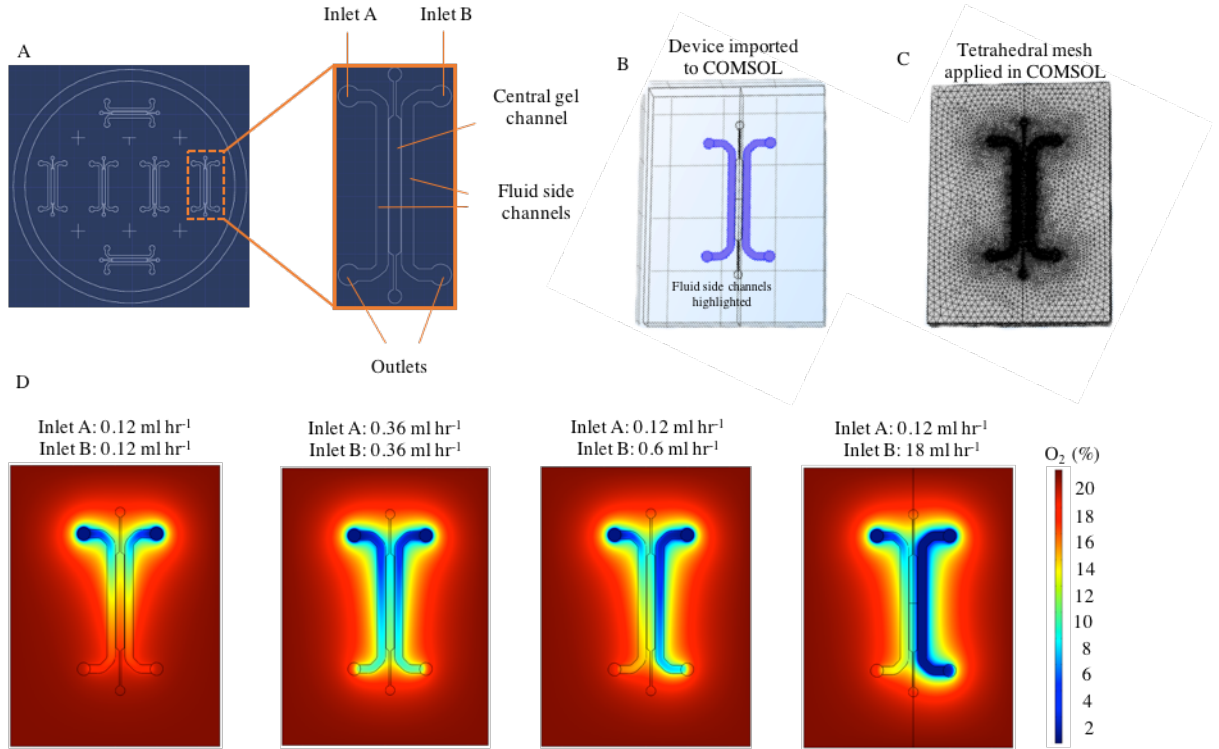


Figure 6.17: **COMSOL oxygen gradient study.** (A) The three channel microfluidic device with the central gel channel separated by $80\ \mu\text{m}$ of PDMS was drawn using AUTOCAD in 2D. The geometry was exported to SOLIDWORKS to generate a 3D drawing and (B) exported again to COMSOL. (C) A tetrahedral mesh was generated on the geometry and (D) oxygen profiles were generated for varying combinations of flow rates into the device inlets.

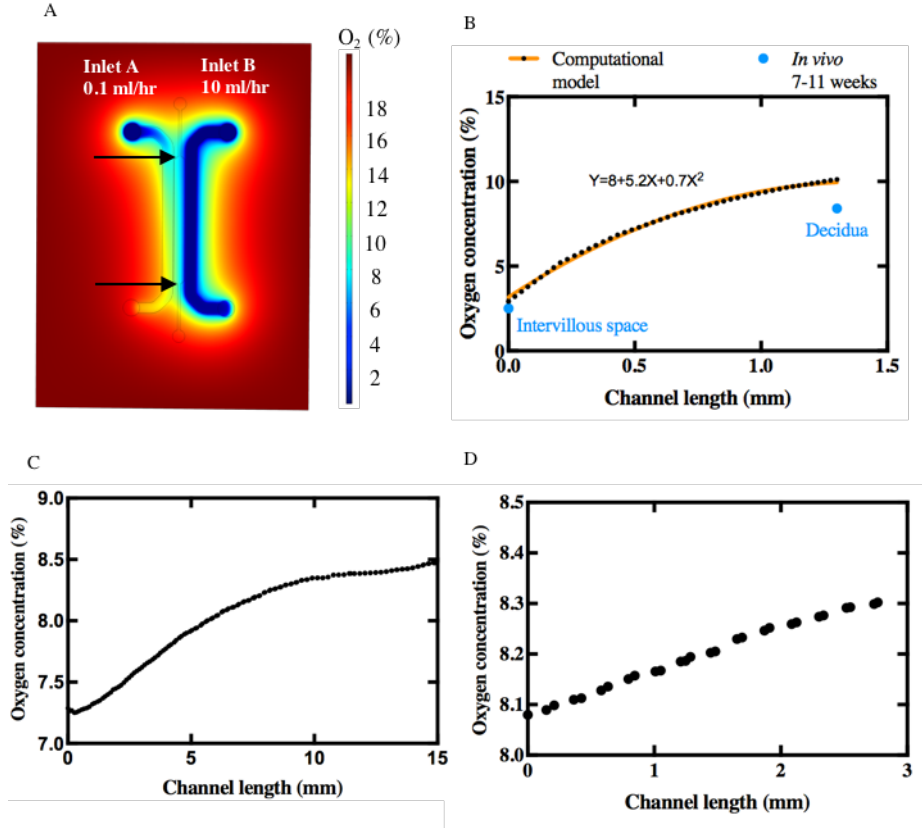


Figure 6.18: **Oxygen gradient across and along central channel.** A) COM-SOL used to model oxygen distribution in the device with flow rates at 0.1 ml/hr and 10 ml/hr in inlet A and inlet B respectively. B) The oxygen gradient estimated across the centre of the gel channel meets the study requirements. The blue points are *in vivo* measurements of the oxygen concentration of the intervillous space and decidua at 2.5% and 8.4% respectively (Jauniaux et al., 2000). C) The oxygen profile along the full length of the central channel, estimated between the two arrows shown in (A). D) The oxygen profile along the length scale of an image taken by a 10 x objective lens, at 2.8 mm.

6.5.9 Gel movement and medium dehydration test with trophoblast cell line

The device was tested with the trophoblast cell line JEG-3 to determine whether the new design prevents gel movement and allows time-lapse tracking of cells.

JEG-3 cells were cultured as described in chapter 3.3 (materials and methods) and embedded in a 1 : 1 mixture of Matrigel : cell solution. Distilled water was perfused through the side channels at 0.1ml/hr and 10 ml/hr for 9 hours at 37 °C and cell movement was imaged during the final hour. There were no leaks from the side channels and there was no visible gel movement during the time-lapse. Because there is no contact between liquid and the gel, there were concerns of dehydration of the cell medium. To prevent dehydration, 200 μ l of medium was pipetted on top of the channel ports containing the gel. This liquid was replenished every 2 h, with care taken not to touch the device with the pipette tip. Embedding the cells in Matrigel diluted with medium further mitigates dehydration issues.

The next step is to conduct experiments with primary extravillous trophoblast cells. However, since August 2017 the surgeon responsible for the surgical pregnancy termination list resigned from Adenbrookes hospital, Cambridge. Despite discussions with representatives of the clinical services at Adenbrookes with the Centre for Trophoblast Research (CTR), it has not been possible to reinstate a surgeon and hence no primary tissue has been available for research purposes. This situation was still ongoing at the time of thesis submission (January 2018).

6.6 Discussion

6.6.1 Simple and economical method for oxygen control in microfluidics

Regulation of oxygen concentration within the microfluidic channels was accomplished using a gas-liquid diffusion system. Liquid was perfused through a sealed vessel containing nitrogen gas and this pre-equilibrated liquid acts as an oxygen sink in the device. Due to this novel design, expensive pressurised gas mixtures are not required and oxygen concentration can be controlled using two methods: (1) the concentration of nitrogen gas in the vessel and (2) the flow rate of pre-equilibrated liquid through the system. The vessel used was a Kilner jar, sealed with a rubber o-ring and vacuum grease. Real-time optical oxygen measurements were used to validate the seal, where in 24 h the increase of oxygen concentration

from 0% at 0 h in the vessel was $0.1 \pm 0.1\%$. The high oxygen permeability of the platinum cured silicone tubing inside the vessel allowed for rapid diffusion and equilibrium with the oxygen set point. Conversely, the barrier in the gas impermeable Viton tubing enabled a stable oxygen concentration entering the device, when monitored for 10 hours using an optical flow through sensor.

For simple control of oxygen concentration, the flow rate of liquid can be adjusted, with gradient generation achieved using two different flow rates for each of the side channels. The high gas permeability of PDMS and the small length scale of microfluidics allows for rapid oxygen diffusion within the device: between the low oxygenated liquid side channels and central gel channel.

The ability to control an oxygen gradient without requiring bulky pressurised gas mixtures mean it is more likely to be used routinely in biological labs. The major cost for experimental set-up are precision syringe pumps, but this is the case for all microfluidics studies. Despite the simplicity in the method for gradient control, there are a number of challenges to setting up this experiment. Troubleshooting and advice for the different steps, including experiments used to validate the oxygen gradient are detailed in table 6.4.

6.6.2 Oxygen gradient validation using optical sensors

In this study, optical oxygen sensing has been utilised for real-time monitoring of gaseous and dissolved oxygen. These sensors do not consume oxygen, which is a major advantage over traditional Clark type electrodes and this is important when sensing at low oxygen concentrations ([Quaranta et al., 2012](#)). The sensors confirmed gas diffusion in the vessel, long term stability in the oxygen concentrations and the high gas permeability of PDMS.

Luminescent thin film sensors were fabricated and integrated into the device to study 2D oxygen distribution in real-time. Several changes were made from the protocol published by Ungerbock *et al.* The solvent used to make the sensor solution was replaced with toluene because of its lower rate of evaporation, and the method of sensor application from knife to spin coating. This resulted in a homogenous application, as confirmed by fluorescent imaging.

An oxygen concentration gradient across the central channel was visualised after perfusion of pre-equilibrated liquid at a low (0.1 ml/hr) and high flow rate (10 ml/hr). Due to the high permeability of PDMS, oxygen diffusion from the microfluidic channels took only seconds. The ability to integrate the sensor underneath the channels is a major advantage over measuring oxygen in the bulk medium as the sensor sits directly underneath the channels.

The sensor film was used to investigate further the increase in dissolved oxygen concentration measured at the inlet ($0.03 \pm 0.01\%$) and outlet ($13.32 \pm 0.019\%$) of the liquid side channels. There was concern that a change in oxygen along the length of the central channel would be conflicting to cells sensing a gradient across. However, a linear regression analysis found no significant difference ($p > 0.5$) in the slope between the experiment with an oxygen gradient and the control. But this was only valid at the length scale of the image analysed ($y=2.8$ mm). COMSOL analysis of the oxygen profile was further evidence that the gradient was more pronounced across the central gel channel than along it, with the change in the oxygen concentration over distance at 5.4 and $0.08 \Delta O_2(\%)/\text{mm}$ respectively. Over the length of scale of an EVT cell (approximately $40 \mu\text{m}$, measured by immunohistochemistry) the change in oxygen concentration was 0.2 and $3.2 \times 10^{-3} \Delta O_2(\%)/40 \mu\text{m}$ EVT cell, for across and along the central channel respectively, which equates to a 68 times difference. This is evidence that the oxygen gradient across the channel, rather than along, is the main driving force for migrating EVT cells, sensing a change in oxygen. Future cell migration experiments should be conducted in the same region (middle of 1.5 cm length central channel) of the device and at the same length scale (10 x objective, $y = 2.8$ mm), to ensure consistency in the oxygen concentration cells are exposed to, along the device.

Improvements in the use of the thin film sensor could be made by adopting Fluorescence Lifetime Imaging (FLIM). FLIM is based on the lifetime of individual fluorophores and the differences in the excited state decay rate from a fluorescent sample. The readout does not depend on concentration of the dye, absorption by the sample and sample thickness (Becker, 2012). Therefore, inconsistencies in sensor film fabrication would be minimised with FLIM. However, FLIM is expensive and requires instrumentation not readily available in biological labs. For simple visual confirmation of a gradient or presence of low oxygen

in microfluidic channels, the set-up described here meets this requirement. It is recommended that the sensor solution is used in batches to avoid variation in the readout between repeats in an experiment.

6.6.3 Computational model confirms oxygen gradient

When gels are embedded in microfluidic devices they are prone to disturbances from liquid flow channels and evaporation of its water content. For small disturbances, in the range of micrometers in hours, fluorescent beads can be combined with a coherent point drift algorithm, as described in chapter 5.3.3.3 for post experiment correction. In this study, Matrigel movement was significant because of the large flow rates required to generate an oxygen gradient leading to detachment from the trapezoidal posts. For this reason, the device was re-designed to confine the gel in closed channels, without contact from flowing liquid. The channel dimensions remained constant and COMSOL was used to select a separation distance of 80 μm between the central and two side channels for optimum oxygen diffusion and device integrity. COMSOL confirmed the experimental results, that oxygen can be controlled using flow rate of liquid entering the device. Flow rates of 0.1 ml/hr and 10 ml/hr in the two side channels were found to generate a gradient that meets the design requirements from 1-3% to 8-10%.

The oxygen validation experiments described in this chapter should be repeated with the re-design. Unfortunately, the oxygen transmitter was on a fixed loan contract from the European Space Research and Technology Centre (ESTEC, Noordwijk, The Netherlands) and the device re-design occurred after it had been returned. However, it was assumed that the validation results can be correlated. The dimensions of each channel remain constant. The change is the removal of PDMS trapezoidal posts with Matrigel in between with a PDMS wall. The diffusion coefficients of Matrigel and PDMS are at the same order of magnitude, $1.5 \times 10^{-9} \text{ m}^2/\text{s}$ and $3.2 \times 10^{-9} \text{ m}^2/\text{s}$ at 37 °C respectively (Colom et al., 2014; Markov et al., 2014).

6.7 Conclusion

In this chapter, a simple and economical method to generate an oxygen gradient within a microfluidic device has been demonstrated. The design requirements outlined in Table 6.1 have been met. The method allows oxygen control without the need of pressurised gas mixtures and gradient generation by controlling flow rates of pre-equilibrated liquid. Optical oxygen sensors confirmed rapid diffusion in the sealed vessel and a stable oxygen concentration of pre-equilibrated liquid entering and exiting the device for at least 10 hours. A gradient was visually confirmed by integrating luminescent thin film sensors, which monitor oxygen directly underneath the central gel channel. COMSOL modelling simulations estimate the gradient to meet the *in vivo* measurements (Jauniaux et al., 2000). The flexibility in gradient generation presented here allows for application in other areas of biological sciences. In particular, cancer tumour hypoxia is thought to lead to dysfunctional vascularization resulting in cell mobility and metastasis (Muz et al., 2015).

6.7 Conclusion

Technique	Problem	Solution
Microfluidics fabrication	Weak bonding between glass slide and PDMS	Clean PDMS and glass surface Check vacuum pressure of plasma cleaner Incubate device at 80 °C for 20 min after plasma cleaning
	PDMS difficult to remove from silicon wafer	Treat wafer surface with silane chemical
	PDMS has not cured	Ensure PDMS base & curing agent are well mixed
	Difficulty in punching holes in PDMS	Replace biopsy punch
Gas-liquid diffusion system	Poor seal on glass vessel - bubble test	Ensure good contact between rubber o-ring and glass rim - no uneven surfaces Apply vacuum grease sparingly on glass rim
	No reduction in dissolved O ₂ concentration	Check for leaks in glass vessel Ensure vessel is well mixed during N ₂ filling Ensure tight connection between port and tubing
Thin film sensor fabrication	Particles/impurities or aggregates visible	Increase dye particle mixing time Sonicate solution for longer
	Uneven film surface after spin coat	Keep solution covered in glass bottle - rapid solvent evaporation Ensure glass is even on chuck
Cell experiment	Medium evaporation	Ensure equal volume on top of the inlet an outlet port of central hydrogel channel
	Defect gradient across central gel channel	Equal length tubing throughout system Ensure syringe pump & glass vessel are all at the same level on microscope stage Check for debris in tubing - flush tubing with deionized water & autoclave before use
	Gel movement in the central channel	Ensure there is equal volume of medium on both central channel ports Batch check Matrigel polymerization

Table 6.4: Troubleshooting set-up for oxygen gradient generation in microfluidics

Chapter 7

Conclusions and future directions

This chapter concludes the thesis. The main results are summarised, with a discussion on the regulation of trophoblast invasion by physical factors at the maternal-fetal interface: tissue stiffness and oxygen. The results from this thesis indicate that these factors may have a role in the regulation of trophoblast invasion *in vivo*. Finally, ideas for future research projects that stem from this work are discussed.

7.1 Conclusions and contributions

Pre-eclampsia, fetal growth restriction and stillbirth are major pregnancy disorders throughout the world. The underlying pathogenesis of these diseases is defective placentation characterised by inadequate invasion of extravillous trophoblast (EVT) cells into the uterine arteries (Brosens et al., 2011). EVT invasion is spatially and temporally finely controlled during the first half of pregnancy. Learning more about what factors influence trophoblast invasion and the cellular and molecular basis of the invasion pathway is essential. Factors that are thought to mediate invasion include decidual cells, cytokines, extracellular matrix (ECM) and oxygen (Abbas et al., 2017; Moffett-King, 2002; Aplin and Jones, 1989; Burton, 2009). In this thesis, tissue stiffness and oxygen were investigated as regulators of trophoblast invasion. To date, there has been no comprehensive study looking at tissue stiffness on invasion and the evidence on oxygen is conflicting.

How cells sense and adapt to their local physical environment is recognised as having important implications in developmental, physiological and pathological contexts (Caballero and Goetz, 2015). Cell migration is known to be influenced by the physical properties of their extracellular environment (Discher et al., 2005). Tissue stiffness, which is defined as the resistance to deformation is one of the many mechanical forces acting on cells. The interest in tissue stiffness on cell fate has led to the characterisation of most tissues in the human body (Vining and Mooney, 2017). This has enabled experiments to be designed that better replicates the physical environment for the particular cell type (Gjorevski et al., 2016).

In this thesis, tissue stiffness of human non-pregnant endometrium, first trimester pregnant endometrium (decidua) and first trimester placenta have been determined using atomic force microscopy (AFM). The stiffness of the decidua basalis was found to be 3-4 times higher than the decidua parietalis in samples matched from the same patient. Decidua basalis is characterised by the presence of EVT cells and tissue sections were confirmed with HLA-G, a marker of the extravillous phenotype. There was no difference between stiffness of non-pregnant and pregnant endometrium, therefore the increase in decidua basalis stiffness is most

7.1 Conclusions and contributions

likely linked to invasion by EVT. This is new evidence of the dramatic change that occurs to the lining of the uterus during pregnancy. The increase in tissue stiffness is hypothesised in part, to be a result of ECM production such as collagen IV by invading EVT cells. Oefner *et al.*, found collagen IV is significantly up-regulated in decidua basalis versus parietalis compared to collagen types I, III and VI and invading EVT cells in the decidua uniquely secrete collagen IV (Oefner *et al.*, 2015). Other explanations include changes to vasculature as a result from remodelling of the spiral arteries with further experiments required in order to localise stiffness measurements.

The result of an increase in tissue stiffness localised to the decidua basalis led to questions on function. Does stiffness have a role in regulating trophoblast invasion? *In vitro* experiments on 2D substrates have shown cells preferentially migrate towards stiffer substrates but as they spread more, the larger number of cell-matrix adhesions per cell are thought to result in slower migration velocities (Isenberg *et al.*, 2009; Lange and Fabry, 2013). In this thesis, primary EVT cells were embedded in interpenetrating networks of Matrigel - alginate to determine if migration speed changes with increase substrate stiffness. Stiffness can be modulated by the concentration of calcium ions used to crosslink alginate. Importantly, this network is in 3D, and ECM concentration and pore size are constant in gels of different stiffness (Chaudhuri *et al.*, 2014). There was no difference in EVT migration in substrates of different stiffness in 3D. Future experiments should investigate EVT migration under stiffness gradients to determine if EVT cells move preferentially towards a soft or stiff matrix.

Matrigel is the most common substrate used to study EVT migration *in vitro*, in 3D. The stiffness of Matrigel was found to vary when measured for 5 batches, with a range from 243.4 Pa to 512.4 Pa, a near doubling of the lower value. This variation is in line with previous studies and is expected as the protein concentration varies from batch to batch (Soofi *et al.*, 2009). In the long term, a synthetic 3D matrix, with defined ECM protein content is needed for the study of EVT invasion (Gjorevski *et al.*, 2016).

Oxygen has long been thought of as a regulator for trophoblast invasion (Graham and Burton, 2004). The development of the extravillous phenotype occurs in a low oxygen environment (20 mm Hg or 2.5%) compared to invasion into the

7.1 Conclusions and contributions

decidua, at an elevated oxygen level (60 mm Hg or 8.4%) (Jauniaux et al., 2000). The evidence to whether oxygen regulates invasion is conflicting, in part because of the differences in the methods used (Caniggia et al., 2000; Fitzpatrick and Graham, 1998). One of the main limitations is the use of trophoblast cell lines that vary in their response to oxygen and exhibit differences at the transcription level to primary EVT (Apps et al., 2011).

In chapter 5, an experiment was designed to study motility of primary EVT cells at oxygen concentrations of 1%, 8% and 21%. The following were adopted to improve on existing work:

1. the placental isolates were stained with an antibody for HLA-G enabling selection of only the extravillous phenotype during analysis
2. EVT cells were embedded in a physiologically relevant matrix and in 3D
3. a low medium volume (200 μ l), which allows for rapid equilibrium to the local oxygen concentration
4. a gradual reduction of oxygen for the 1% condition over 3 h, to reduce stress on EVT cells
5. an incubation time of 24 h to enable time for EVT cells to sense and adapt to the local oxygen concentration

A point set registration algorithm was used to find the spatial transformation of stationary fluorescent beads between two points, 0 h and 24 h. The root mean squared displacement (RMSD), which is the average distance a cell moves in a given time interval was quantified. There was no difference in the motility of EVT cells at 1% compared to the physiological control of 8% and 21%. Although there was a trend of increased motility as oxygen decreases when the RMSD of all 6 patient samples were averaged, this trend was only seen for two patient samples. This result opens the debate that oxygen may have no effect on trophoblast motility.

It is difficult to compare this finding to literature, because of the wide differences in experimental procedure and cell types used. Certainly, the use of primary EVT and selecting of only the extravillous phenotype is the main strength of this

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study and goes beyond previous work. However, it is important to be clear that the measure of RMSD is not one of cell speed and more time points are needed to extend this work. Analysis of the RMSD of EVT cells imaged continuously (every 20 min for 6.7 h) indicates EVT motion in 3D does not follow random walk and is constrained. This was evident by a plateau of the RMSD vs. time within 20 min. This result is in line with Wu *et al.*, who hypothesises 3D migration is influenced by the heterogeneity in the density, pore size, fibre thickness and matrix stiffness of local ECM Wu *et al.* (2014).

The next step in the study of oxygen as a regulator of trophoblast invasion, is to determine whether invasion is directed. In chapter 6, an *in vitro* model using microfluidics with an oxygen gradient that approaches the gradient during the first trimester of pregnancy was designed and validated. A gradient was generated by perfusing pre-equilibrated liquid into two side channels of a microfluidic device, with a gradient generated in the central channel containing cells. Microfluidics has been used previously to study EVT invasion under gradients of the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) (Abbas *et al.*, 2017). Importantly, the directionality of cells can be tracked in 3D and the micron sized channels means only a few thousand cells are needed.

A simple and economical gas-liquid diffusion system was developed to equilibrate liquid to low oxygen, using a gas tight glass vessel and a feed of nitrogen gas. Due to this novel design, expensive pressurised gas mixtures were not required and oxygen concentration could be controlled using two methods: (1) the concentration of nitrogen gas in the vessel and (2) the flow rate of pre-equilibrated liquid through the system. Optical oxygen sensors were used to confirm the vessel was air tight, efficient diffusion in the vessel, long term stability in the oxygen concentration in the vessel and the high gas permeability of PDMS. Optical oxygen sensors provide real-time measurements and do not consume oxygen, unlike traditional Clark type electrodes. For visualisation of the gradient, a luminescent thin film sensor was fabricated and integrated into the device. A gradient of oxygen was confirmed across the width of the central cell containing channel. The microfluidic device had to be redesigned to maintain gel stability during an experiment and COMSOL computational modeling was used to estimate that the gradient still meets the *in vivo* measurements. The next step is to use this

7.2 Future research directions

system with primary EVT cells to determine whether trophoblast migration is directed. This experiment could explain why EVT cells migrate towards arteries rather than veins. Or whether changes in oxygenation between the decidua and myometrium explain why the EVT stop invading.

In conclusion, physical factors at the maternal-fetal interface have a role in the regulation of trophoblast invasion. The change in tissue stiffness in the decidua as a result of implantation of the placenta is further evidence of the dramatic change that occurs in the uterine wall during pregnancy. Further experiments are required to determine whether stiffness is a regulator of trophoblast invasion. The role of oxygen in regulating trophoblast invasion has been a subject of much debate within the placenta field. In part, because of the differences in experimental method and the continual use of cell lines. This investigation suggests that oxygen has no effect on EVT motility in 3D and *in vitro*. Importantly, only cells that have the HLA-G antigen, a signature of the extravillous phenotype were analysed. Investigating how EVT cells respond to a gradient of oxygen will enable understanding to whether EVT cell migration is directed towards increasing or decreasing oxygen concentrations. Microfluidics is a suitable tool to study cell migration under oxygen gradients in 3D and real-time.

7.2 Future research directions

7.2.1 Modular synthetic hydrogel networks to study trophoblast migration

Matrigel was used in this study as the 3D matrix to study trophoblast migration. The ECM protein and growth factor content of Matrigel is known to vary between batches (Soofi et al., 2009). These animal derived gels are challenging to modify and tailor to specific tissue types. There is a need for a modular, synthetic hydrogel network that replicates the stiffness of the decidua basalis found in this study and ECM content determined in literature (Aplin and Jones, 1989; Oefner et al., 2015). Recently, Gjorevski et al., published a protocol developing a modular synthetic hydrogel network for intestinal stem cell (ISC) and organoid

culture (Gjorevski and Lutolf, 2017). These gels are polyethylene glycol (PEG) based and are enriched with ECM proteins that are known to enhance ISC survival and proliferation. The stiffness can be controlled with the concentration of PEG precursors as a function of overall PEG content. The adoption of a well-defined matrix would improve the reliability of studies that culture EVT cells using different batches of Matrigel.

7.2.2 Proteomic analysis on ECM proteins in the decidua

In this study, stiffness in the decidua was found to increase by fivefold as a result of the implanting placenta. It was hypothesised that this increase was in part due to secretion of collagen IV by invading EVT cells. This hypothesis was based on immunohistochemistry data, which shows collagen IV is significantly up-regulated in decidua basalis versus parietalis compared to collagen types I, III and VI (Oefner *et al.*, 2015). However, the work done by Oefner *et al.*, is a snapshot of the ECM composition and the full extent of the differences remains unknown. A quantitative proteomic analysis is needed to characterise the ECM proteins between decidua basalis and parietalis. Because ECM in the decidua is dynamic, undergoing constant remodelling, this analysis should be done on decidua at different stages during the first trimester. Decellularization and ECM protein enrichment of the decidual tissue are required. A number of protocols have been published to digest ECM enriched protein samples into peptides, involving incubation in buffers of different pH and detergent concentrations (Naba *et al.*, 2015). Peptides are then labelled with tags for quantitative proteomic analysis and analysed using mass spectrometry (Naba *et al.*, 2017). ECM proteins can then be identified and their abundance quantified.

7.2.3 Tissue stiffness of non-pregnant endometrium in healthy vs. infertile women

This work could be extended further by determining tissue stiffness of non-pregnant endometrium in healthy women, which would be compared to data determined in this study, from women who have infertility issues. Differences in

tissue stiffness, could indicate a role for mechanics in individuals that suffer recurrent miscarriage. However, there may be challenges in obtaining endometrium biopsies from healthy, fertile women. One option is from patients with suspected endometrial cancer, but this is a disease typical of older, postmenopausal women (Garg and Soslow, 2014).

7.2.4 Understanding the mechanisms of directed migration towards oxygen

The microfluidic oxygen gradient system developed in this study, would allow biologists, placental and cancer, to investigate the mechanisms by which cells sense and move towards oxygen. Examples would be to knock down hypoxia-inducible factors (HIFs) as a hypoxia sensing transcription factor using interference RNA (Ono et al., 2006) or CRISPR/cas9 (Ran et al., 2013) and observe whether there is an alteration in response. Cells with and without the knock down gene could be seeded in the same device and identified by cell tracker dyes at different emission intensities. In recent years, CRISPR (clustered regulatory interspaced short palindromic repeats) in combination with cas9 (CRISPR associated protein 9) has emerged as a cutting edge genome editing tool to knock down microRNA *in vitro* and *in vivo* (Chang et al., 2016). Other targets include transforming growth factor beta (TGF- β), the metalloproteinases (MMPs) etc.

Appendix A

Appendix

Command prompts to run 'blob' detection and coherent point drift algorithm as described in materials and methods, chapter 3.7.2.2. An example code is detailed below for an image taken at 0 h and 24 h, named 'Image1_0h.tif' and 'Image1_24h.tif' respectively. This code should be entered into terminal.

- Call on the blob detection algorithm:
 - `cd ~/Documents/CPD/blob`
 - `git pull`
- Run the blob detection algorithm. Set threshold point identification at 40 (unitless):
 - `python3 blob.py find ~/Documents/CPD/Image1_0h.tif -size 2 3 -threshold 40 >`
 - `~/Documents/CPD/Image1_0h.csv`
 - `python3 blob.py find ~/Documents/CPD/Image1_24h.tif -size 2 3 -threshold 40 >`
 - `~/Documents/CPD/Image1_24h.csv`
- View the point detection:
 - `python3 blob.py plot ~/Documents/CPD/Image1_0h.tif`

-
- `~/Documents/CPD/Image1_0h.csv -axes 0 1`
 - `python3 blob.py plot ~/Documents/CPD/Image1_24h.tif`
 - `~/Documents/CPD/Image1_24h.csv`
 - Change directory to CPD algorithm:
 - `cd ~/Documents/CPD/coherent-point-drift`
 - `git pull`
 - Run CPD alignment:
 - `python3 align.py align ~/Documents/CPD/Image1_0h.csv -axes 0 1`
 - `~/Documents/CPD/Image1_24h.tif`
 - `~/Documents/CPD/Image1_24h.csv -w 0.1 -format pickle >`
 - `~/Documents/CPD/Image1_0h/Image1_24h.pickle`
 - View CPD alignment visually:
 - `python3 align.py plot ~/Documents/CPD/Image1_0h.csv -axes 0 1`
 - `~/Documents/CPD/Image1_24h.tif`
 - `~/Documents/CPD/Image1_24h.csv`
 - `~/Documents/CPD/Image1_0h/Image1_24h.pickle -axes 0 1`
 - Save results as .mat (matlab) file:
 - `python3 align.py align`
 - `~/Documents/CPD/Image1_0h.csv -axes 0 1`
 - `~/Documents/CPD/Image1_24h.tif`
 - `~/Documents/CPD/Image1_24h.csv -w 0.1 -format mat >`
 - `~/Documents/CPD/Image1_0h/Image1_24h.mat`

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