Development of 2 dimensional strain bioreactor for the study of effects of mechanical stimulation oesophageal cells

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DEVELOPMENT OF
2 DIMENSIONAL STRAIN BIOREACTOR
FOR THE STUDY OF
EFFECTS OF MECHANICAL STIMULATION ON
OESOPHAGEAL CELLS

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Abstract

The number of global occurrences for oesophageal cancer ranks 6th among men and 8th among women in the year 2002. However, epidemiological studies have shown that specific ethnic groups, particularly Asians and Africans are more prone to developing oesophageal cancer.

Although the survival rates for oesophageal cancer have improved significantly over the last half a century, the overall survival rate is still considered low. Current methods of oesophageal cancer management are far from ideal. Even with chemo- and radio-therapy combining with surgical treatments providing the highest opportunity of success, the quality of life of the patient after treatment is usually greatly reduced. An alternative treatment strategy would be to tissue engineer a clinically functional oesophagus for the use of organ transplantation.

As previous research have shown that mechanical stimulation is able to induce positive effects on cellular proliferation and expression of cell phenotype, this project proposes to study the effects of mechanical stimulation on oesophageal cells. It is hoped that the knowledge gained from this study is able to facilitate the tissue engineering of a clinically functional oesophagus.

In the course of candidature, a bioreactor system capable of providing two-dimensional strain was designed and fabricated. Additionally, a suitable substrate was successfully fabricated from Chronoflex® AL-80A polyurethane pellets to provide the bioreactor actuation membrane and subsequently surface modified via oxygen plasma discharge. The fabricated polyurethane membrane was analysed with a Thermo Gravimetric Analyzer to detect the presence of any residual cytotoxins.

A primary cell line of oesophageal smooth muscle cells was isolated from a sample of porcine oesophagus and characterised by immunocytochemical staining with cytoskeletal protein markers. The isolated porcine oesophageal smooth muscle cells were also used to conduct cell proliferation and cell attachment experiments on the

oxygen plasma treated polyurethane membrane to ensure that it is biocompatible and non-cytotoxic.

Oesophageal smooth muscle cells were mechanically stimulated by the two-dimensional strain bioreactor at 0% (control), 2% and 5% radial strain and were analysed on the effects of mechanical stimulation on the cell alignment, cell proliferation as well as their phenotype expression as a result of two-dimensional strain.

Oesophageal smooth muscle cells were aligned both perpendicularly and parallel to the radial strains with increasing proportion of parallel alignment as the tangential strain increased. High magnitude of mechanical strain was found to induce massive loss of cells either through apoptosis or cell detachment. Cytoskeletal protein analysis revealed that mechanical stimulation induces cellular remodelling resulting in an initial down-regulation of cytoskeletal differentiation markers before being progressively up-regulated over time.

In conclusion, cyclic two-dimensional mechanical strain is demonstrated to be a suitable external stimulus to biomimic the physiological state of a normal functional oesophagus thereby inducing cell/tissue remodelling of the oesophageal smooth muscle cells by its cellular reorientation as well as the induction of cellular differentiation closer to the smooth muscle cells found *in vivo*.

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Nomenclature

Letters

L	Original length
P	Pressure of fluid acting on bioreactor membrane
r	Radial co-ordinate of bioreactor well
r'	Radial co-ordinate of bioreactor well due to deformation
R	Well diameter of bioreactor
t	Thickness of bioreactor membrane
V	Volume of actuation fluid
ΔV	Change in volume of actuation fluid to cause a fixed maximum vertical deflection
y	Local vertical deflection of the bioreactor membrane along the radial co-ordinate, r
$y_{\max}$	Maximum vertical deflection of the bioreactor membrane along the central axis of the bioreactor well

Greek Letters

α	Angle between central axis and radius of curvature at bioreactor well edge
β	Angle between central axis and radius of curvature at any point along radius of bioreactor well
Δ	Change in variable
δ	Small change in variable
δr	Small change in radius
$\delta \theta$	Small change in angle
ϵ	Strain
ϵ_r	Radial strain
ϵ_θ	Tangential strain
ϕ	Angle of deflection of bioreactor membrane
ρ	Radius of curvature of the bioreactor membrane for any given maximum vertical deflection

σ_r	Radial stress
σ_θ	Tangential stress
θ	Angular co-ordinate of bioreactor well

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1 INTRODUCTION

1.1 Background

From the data obtained from GLOBOCAN 2002, it is revealed that the occurrence of oesophageal cancer ranks 6th among men and 8th among women in the year 2002 [1]. Additionally, the mortality rate for oesophageal cancer is exceptionally high compared to other types of cancer [1, 2].

Although the survival rate of oesophageal cancer has improved significantly over the last 50 years, the overall survival rate is still considered low [2]. Epidemiological studies have shown that specific ethnic groups are more prone to developing oesophageal cancer, with Asian and African populations on the top of the list [1-3]. Oesophageal cancer occurs 10 to 100 times more frequently in these ethnic groups when compared with the United States [2, 3]. Thus, the area extending across from Northeast China through central Asia to the Middle East region is also referred to as the “Asian Oesophageal Cancer Belt” [3].

Currently, there are several means of treatment available for the management of oesophageal cancer with surgical resectioning as the most common method [3-6]. However, surgical resectioning usually may result in a significant decrease in the quality of life after the surgical procedure [7, 8].

In cases where surgical treatment is not possible, artificial devices such as the extracorporeal artificial oesophagus and the intraoesophageal stent have been used to manage the disease [9]. However, they are only palliative devices and do not in themselves offer a long term solution to the problem. Also, the usage of artificial materials thought to be biocompatible, such as stainless steel, nylon, polyethylene, Dacron® and Teflon® [9-11], as direct oesophageal replacements have all ended in failure due to host immune response [9, 11].

Even though there are cases of limited success using artificial materials such as polymers, metals and ceramics as tissue or organ replacements [12], the overall biocompatibility of these artificial materials are still far from ideal even with

processes such as surface modification in the case of polymers [11, 13]. Varying degrees of immune response would still be induced when these materials are placed inside the body.

In addition, as these materials would not be able to remodel themselves to adapt to the changes in activity level as required by the body and also growth in the case of a growing child or to replicate the entire range of functions possible by the original tissue or organ. These artificial prostheses are susceptible to wear and/or corrosion, which limits their service life [12]. Thus, the usage of artificial materials as tissue or organ replacements only serves as an intermediate solution.

However, with the rapid development and progress of life sciences such as cell technologies and the application of traditional engineering knowledge in the context of biology, the field which is known today as tissue engineering has emerged as a possible solution to meet the needs of surgical reconstruction [11, 14] and the production of fully functional and living biological replacements within a laboratory may one day become a reality [10, 11, 14, 15].

Currently, there are two main approaches to the tissue engineering, which are *in vivo* regeneration and *in vitro* reconstruction [11, 14, 16-18]. *In vivo* regeneration requires the use of tissue inducing substances such as growth factors or/and the presence of a scaffold to mobilise cells from the nearby native tissue to populate and proliferate within the scaffold. The rate of degradation of the scaffold is critical to the success of *in vivo* regeneration. Rapid degradation of the scaffold would result in the disintegration of the cell/scaffold construct, whereas slow degradation of the scaffold might affect the regeneration process adversely [10, 17].

The *in vitro* reconstruction approach to tissue engineering requires the three dimensional assembly of cells and scaffolds through the use of biochemical and biomechanical signals to form a living and functional tissue within a controlled environment provided by a tissue engineering bioreactor. Cells have to be harvested from either autologous, allogeneic or xenogeneic sources [11, 15, 18-21] and seeded onto a scaffold or extracellular matrix (ECM) made of naturally occurring materials such as collagen or synthetic polymers such as polyglycolic acid (PGA), polylactic

acid (PLA) [10, 11, 15, 20, 22-24] or even acellular scaffold from xenogeneic sources [10, 25-27]. Figure 1 shows the process of tissue engineering via the *in vitro* approach.

Figure 1. Tissue engineering via *in vitro* reconstruction (Vacanti and Langer,1999) [21]

Thus, in order for three dimensional cell/scaffold constructs to be successfully grown *in vitro*, tissue specific bioreactors will have to be designed and constructed to facilitate the growth of specific functional tissues. Additionally, in order to increase our understanding on the effects of external stimulation on cellular response, new bioreactors will have to be designed to facilitate such studies in a controlled environment.

In vitro studies of cellular response to mechanical stimuli conducted previously have shown that mechanical stimulation is able to improve the structural and functional properties of the cell/scaffold constructs [28, 29] and also induce favourable effects such as the increase in the rate of proliferation and extracellular matrix synthesis [29] and the regulation of other cellular function such as phenotype expression, morphogenesis and cellular alignment/orientation [29-33].

However, in most of these previous studies the cell type used are vascular or cardiac cell types [34] and hence may not be representative of the cell types found in the

oesophagus. In addition, even though it has already been established that mechanical stimulation does induce favourable cellular response, little is known about the exact optimal conditions for parameters such as strain magnitude, frequency and duty cycle [29].

In order to advance the effort to achieve a tissue engineered oesophagus, this study will focus on enteric cells from the alimentary tract, which is formed by the oesophagus, stomach and intestines, specifically porcine oesophageal cell types and their cellular response to externally applied mechanical stimuli via two-dimensional strain.

1.2 Objective

The objective of this project is to investigate the effects of mechanical stimulation by two-dimensional strain on oesophageal cell types in cell/scaffold constructs *in vitro*.

As two-dimensional mechanical strain is experienced by the oesophageal wall during ingestion as a result of a food bolus, it is hoped that by studying the effects that two-dimensional mechanical strain have on cellular response, sufficient knowledge can be acquired to facilitate the aim of tissue engineering a clinically functional oesophageal replacement as a treatment option for oesophageal cancer.

1.3 Scope

The scope of this project includes the design and fabrication of a bioreactor capable of imparting two-dimensional mechanical strain to cell/scaffold constructs *in vitro*. This is to facilitate the study of the effects of two-dimensional mechanical strain on oesophageal cell types. A suitable substrate for use as the bioreactor actuation membrane is fabricated using Chronoflex® AL-80A polyurethane pellets and evaluated for its biocompatibility and non-cytotoxicity.

A primary cell line of oesophageal smooth muscle cells is isolated from porcine oesophageal tissues. This line has been shown to consist of functional cells by immunocytochemical staining.

Surface modification of the bioreactor actuation membrane is conducted using oxygen plasma discharge to reduce its hydrophobicity. Cell proliferation and cell attachment experiments are conducted with the oxygen plasma treated bioreactor actuation membrane to evaluate its suitability as a two-dimensional scaffold for the study of two-dimensional mechanical strain on oesophageal smooth muscle cells.

The oesophageal smooth muscle cells are mechanically stimulated with 2% and 5% radial strain for 420 cycles a day at 5 cycles per minute and compared against a 0% radial strain control group. The cellular responses are then evaluated from the cell alignment, cell proliferation and cytoskeletal protein analysis.

2 REVIEW OF LITERATURE

2.1 Oesophagus

2.1.1 Anatomy of the Oesophagus

The oesophagus is a muscular tube of about 25 cm in length [35-38]. The oesophagus passes food from the pharynx to the stomach. It passes through the diaphragm before terminating at the gastric sphincter. It can be divided into 3 regions, the cervical, thoracic and abdominal, as shown in Figure 2.

Figure 2. Position of main anatomical elements of the oesophagus with respect to other organs (Iazetti *et al.*, 2005) [36]

The Upper Oesophageal Sphincter (UOS) and the Lower Oesophageal Sphincter (LOS) are located above and beneath the oesophagus respectively. The oesophageal sphincters serve to maintain the oesophageal lumen at a vacuum by acting as valves.

The Upper Oesophageal Sphincter excludes air during respiration and the Lower Oesophageal Sphincter excludes gastric contents from refluxing into the oesophagus [37, 39].

The oesophageal wall can be categorised by 4 layers, namely the mucosa, submucosa, muscularis externa and the adventitia. Figure 3 and Figure 4 show the various layers of the oesophageal wall.

Figure 3. Structure of the oesophageal wall (Orlando, 1997)[39]

Figure 4. Microscopic structure of the oesophageal wall (Marieb, 1995) [35]

The mucosa consists of the stratified squamous epithelium, the lamina propria and the muscularis mucosa. Below the mucosa, there is the submucosa layer which consists of areolar connective tissue. Mucus-secreting oesophageal glands can be found within the submucosa. When not involved in the transportation of food, the mucosa and submucosa layer are thrown into longitudinal folds as shown in Figure 4 (a) and its lumen is empty and collapsed [35, 39].

However, during peristalsis, where a food bolus is in transit through the oesophagus, the mucosa and sub mucosa are completely stretched and the compression of the oesophageal glands by the food bolus cause mucus to be released, aiding food transport by lubricating the oesophageal wall [35].

The muscularis externa can be found beneath the submucosa. The muscularis externa consists of the circular muscle layer and the longitudinal muscle layer. The circular muscle layer is aligned circumferentially while the longitudinal muscle layer is aligned vertically along the length of the oesophagus. However, it has been previously

observed that in vascular muscle layers *in vivo*, the smooth muscle cells are actually not completely circumferential but aligned in a spiral (helical) arrangement with a small pitch angle [31].

Additionally, the ratio of skeletal muscle cells to smooth muscle cells in the muscularis externa varies along the length of the oesophagus. Although composition of skeletal muscle cells to smooth muscle cells cited are different from source to source, there is general consensus that skeletal muscle cells are predominant at the cervical part of the oesophagus, gradually decreasing in proportion as it becomes more distal from the Upper Oesophageal Sphincter until it reaches the abdominal part where it is predominantly smooth muscle cells [35, 39].

Unlike the rest of the gastrointestinal tract (alimentary canal), the oesophagus does not have a serosa as its exterior layer but has an adventitial layer instead [35, 39]. The adventitia is composed entirely of loose fibrous connective tissue which serves to blend with the surrounding structures along its route from the pharynx to the stomach [35].

2.1.2 Barrett's Oesophagus

Barrett's Oesophagus, first reported by Norman Barrett in 1957, is a condition where the stratified squamous epithelium normally found at the inner wall of the oesophagus (mucosa layer) has been replaced by columnar epithelium cells normally found at the intestinal walls [39, 40]. The presence of this condition occurs at the lower oesophagus nearest to the stomach.

Figure 5 shows the histological features of a normal oesophageal wall which is lined with a partially or nonkeratinized stratified squamous epithelium [35, 39]. From the endoscopic view as shown in Figure 6, it can be observed that the red tissue in the background is the Barrett's tissue (columnar epithelium lined oesophageal wall) and the whitish- pink tissue in the foreground is the normal oesophageal wall lined with stratified squamous epithelium. Also, from Figure 4 (b), the arrow shows the abrupt transition from the stratified squamous epithelium of the oesophagus to the columnar epithelium found in the stomach. In the case of the Barrett's oesophagus, this

transition point is moved further up within the oesophagus instead of at the oesophageal-stomach junction [35].

Figure 5. Histology of normal oesophagus (Orlando, 1997) [39]

Figure 6. Endoscopic view of Barrett's Oesophagus (<http://www.fhcrc.org/science/barrets/plain.htm>)

However, the confirmation of this condition can only be made after a biopsy, where a sample of the suspected Barrett's tissue is obtained from the patient's oesophagus and subjected to histological investigation [39].

Currently, the Barrett's oesophagus is widely considered to be a common acquired condition which develops as a result of chronic Gastro-oesophageal Reflux Diseases (GORD) [40].

GORD is a disorder whereby there is recurring reflux of the stomach contents back up into the oesophagus [41]. This reflux can be due to a variety of reasons ranging from over eating and drinking, extreme obesity, pregnancy to even a pathological condition such as hiatus hernia, a structural abnormality where the top part of the stomach protrudes slightly above the diaphragm [35].

For a patient with the condition of hiatus hernia, the stomach is protruding above the diaphragm and the Lower Oesophageal Sphincter (LOS) is no longer reinforced. Thus, the likelihood of gastric juices refluxing back into the lower oesophagus is increased. This reflux causes heartburn. If the refluxes are frequent and prolonged, the inflammation of the oesophagus, *oesophagitis*, and oesophageal ulcers may develop as a consequence.

When exposed to such constant refluxes, the normal oesophageal stratified squamous epithelium may be damaged by the highly acidic gastric contents. However, as the damaged oesophageal stratified squamous epithelium are still exposed to the abnormal environment of chronic gastroesophageal reflux during the healing process, intestinal metaplasia occurs and columnar epithelium cells grow back in place of the normal stratified squamous epithelium cells to line the lower oesophageal wall [40, 42].

Once these columnar epithelium has replaced the damaged region of stratified squamous epithelium cells, the frequency of discomfort as well as intensity experienced by the patient may decrease as the metaplastic cells are less sensitive to the gastric reflux contents as compared to the normal oesophageal tissue.

A patient with intestinal metaplasia is at increased risk of developing oesophageal cancer. Figure 7 shows the inner lining of the oesophagus undergoing a sequence of metaplastic changes of which the inner lining of the oesophagus develops from the normal stratified squamous epithelium cells into Barrett's oesophagus tissue before finally resulting in invasive carcinoma cells.

Figure 7. Metaplastic process of stratified squamous epithelium cells into invasive carcinoma cells (<http://pathology2.jhu.edu/beweb/Definition.cfm>) [42]

It may be believed that the replacement of normal tissue with specialised columnar epithelium would result in fewer symptoms of gastro-oesophageal reflux. However, it does not indicate the absence of intestinal metaplasia at the lower oesophagus. Hence, patients who have been diagnosed with Barrett's oesophagus have a risk of developing oesophageal cancer that is 30 to 125 times higher than that of the general population [42].

2.1.3 Oesophageal Cancer

For cancer of the oesophagus, there are 2 main types, squamous cell carcinoma and adenocarcinoma.

Squamous cell carcinoma is the cancer of the squamous epithelium. As the whole inner wall of the oesophagus is lined with squamous cells, this type of oesophageal cancer has the potential to occur anywhere along the oesophagus and is most often found in the upper two thirds of the oesophagus, closer to the pharynx [5].

Adenocarcinoma is defined as the cancer of cells that lines certain internal organs and exhibit glandular structures and functions [43]. In the case of the oesophagus, adenocarcinomas usually develop in the lower third of the oesophagus, closer to the stomach. The development of adenocarcinomas in the lower oesophagus is not a sudden transformation in cell/scaffold morphology but a result of progressive metaplastic transformation of the normal squamous epithelium to high-grade dysplasia in the lower third of the oesophagus possibly due to long term conditions such as gastro-oesophageal reflux [5, 38, 44].

The incidence of oesophageal cancer occurs more often in men than women [1-3, 5], and it is more likely to occur in the older population [2, 3, 5]. Epidemiological studies have indicated that certain ethnic groups such as Africans and Asians are more likely to develop oesophageal cancer with the number of incidences of oesophageal cancer in Asia accounting for at least 75% of all reported incidences [1-3].

Although the survival rate for oesophageal cancer has significantly improved over the past 30 years, the overall survival rate after being diagnosed with oesophageal cancer

is still low with less than 50% surviving after 2 years and less than 20% after 5 years [2, 3]. Regular endoscopic surveillance carried out on high risk groups has produced conflicting opinions on the cost effectiveness of such procedures although early detection and treatment of the cancer does increase survival rates [40, 45, 46].

Apart from gender and age, factors such as alcohol and tobacco consumption also increase the risk of developing oesophageal cancer. Dietary habits such as a high-fat, low protein and low calorie diets haven been shown to increase the risk of developing oesophageal cancer, in addition to the consumption of (thermally) hot food and drinks and other diets that might promote gastro-oesophageal reflux [3, 47].

Currently, oesophageal cancer can be treated with either surgical resectioning or chemotherapy with radiation therapy (also known as chemoradiation therapy). However, the suitability of these treatments depends on the stage, size and location of the cancerous cells. A combination of chemotherapy and radiation therapy is often used in conjunction with surgical treatments to first reduce the size of the tumour before removing it from the body surgically [3-5].

Surgical procedures that are currently used for the treatment of oesophageal cancer are oesophagectomy and oesophagogastrrectomy.

Oesophagectomy involves the surgical removal of affected portions of the oesophagus as well as the nearby lymph nodes and reconnecting the remaining healthy portion of the oesophagus with either the stomach or the lower part of the oesophagus [5, 6].

Oesophagogastrrectomy involves the removal of affected areas of the oesophagus, nearby lymph nodes and also the upper part of the stomach. The remaining healthy portion of the upper oesophagus is then connected directly to the modified stomach and the position of the stomach may have to be shifted upwards to allow such a reconnection. Due to the removal of the Lower Oesophageal Sphincter (LOS), there is no longer any device to stop the reflux of gastric juices up into the oesophagus. A vagotomy may be required to sever the vagus nerve to reduce the secretion of acid in the stomach [4].

In the case where a large portion of the oesophagus has to be surgically removed due to the extent of the spread of the disease, an oesophageal replacement is required to reconnect the top portion of the oesophagus to the rest of the gastrointestinal tract. Several strategies are currently used for the surgical replacement of the diseased portion of the oesophagus. The stomach can be surgically remodelled into a tube (i.e. tubularised) and be directly reconnected to the remaining oesophagus. Alternatively, autologous grafts such as the colon interposition [3, 4, 6, 48] or jejunal interposition [3, 6] can be used to connect the remaining oesophagus to the stomach.

Colon interposition is the use of a section of the colon to connect the remaining oesophagus with the stomach to serve as a conduit whereas in jejunal interposition, the small intestine is used.

Another method of connecting the remaining oesophagus to the stomach is gastric transposition. In gastric transposition, the diaphragm is widened to allow the stomach to pass through it to be reconnected back with the remaining oesophagus [48].

Although surgical procedures may be used to manage oesophageal cancer with varying degrees of success, the patient would have to put up with the risk of developing surgical complications such as anastomic leakage and stenosis [11]. The quality of life after surgical would also be significantly affected and the patient would have to adjust to new lifestyle arrangements such as taking a liquid or softer diet as well as learning new breathing techniques to keep their lungs open [5, 7].

In addition to the traditional forms of cancer treatments mentioned above, other forms of therapy such as laser therapy, photodynamic therapy and electrocoagulation (i.e. the use of an electric current to kill cancer cells) are also used in the treatment of oesophageal cancer [3-6].

Apart from the use of autologous replacement in the treatment of oesophageal cancer, the use of artificial materials as oesophageal replacement has also been previously investigated. This use of oesophageal prosthesis is only used in the treatment of oesophageal cancer when an autologous replacement is not possible. Previous usage of artificial oesophageal replacements have been largely unsuccessful, with the

exception of the extracorporeal-type oesophagus and the intra-oesophageal stent tube, due to anastomosis dehiscence (i.e. leakage of the surgical connection due to imperfect suturing or rupture) which results in fatal infections and also the dislodgment and migration of the prosthesis and even stenosis [9].

The extracorporeal artificial oesophagus is a device which connects the cervical oesophagus to the stomach via a latex rubber or silicone tube which extents out of the body to the front of the chest as shown in Figure 8 [9].

Figure 8. An extracorporeal artificial oesophagus (Nakamura and Shimizu,2000) [9]

The other type of artificial oesophageal prosthesis is the intraoesophageal stent, which is basically a plastic or rubber tube. This device is used only when surgical procedures are not possible for the treatment of oesophageal cancer and the stent is placed at the stenotic region of the oesophagus to keep the oesophageal lumen open and prolong the function of the oesophagus. Thus, this procedure only provides temporary relief to the patient and is not a complete treatment in itself. Figure 9 shows the intraoesophageal stent and its function [9].

Figure 9. An intraoesophageal stent and its usage (Nakamura and Shimizu,2000) [9]

Due to the shortcomings of current treatments, and in order to achieve a long term solution and effectively manage oesophageal cancer, tissue engineering may hold the key towards enabling the development of a fully functional and living biological prosthesis as a replacement for the diseased organ.

2.2 Tissue Engineering

2.2.1 Tissue Engineering: An Overview

Tissue engineering is described as “an interdisciplinary field that applies the principles and methods of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function” [11, 14, 21]. It makes use of cells, scaffolds or extracellular matrix (ECM) and signals to be assembled in a three-dimensional manner over time to form tissues or/and organs [10, 11, 15, 23].

With the shift of strategies for reparative medicine from the substituting of one body part for another (autologous grafting) within the same individual, to transplanting organs from another individual (allogeneic grafting) and even from other animals (xenogeneic grafting), the use of artificial materials for use in first, non-vital and later, in vital life sustaining applications was subsequently sought after. Currently, attempts are being made to use living cells to restore, maintain or enhance the function of tissues and organs, which is the definition of tissue engineering today [10, 15, 22] and almost all tissue/cell types are being investigated for tissue engineering purposes [11, 14, 21].

However, the final goal of tissue engineering is to “restore function through the delivery of living elements which become integrated into the patient” [11, 21]. Thus, in order to achieve success in the multi-disciplinary field of tissue engineering, medical practitioners, scientists and engineers have worked together in a collaborative manner in an attempt to find an answer to this multidisciplinary problem [10, 11, 15, 22].

2.2.2 Types of Tissue Engineering

Currently, tissue engineering can be classified into two main types. They are *in vivo* guided tissue regeneration, and *in vitro* reconstruction [11, 14, 16-18].

In vivo regeneration relies on the use of a scaffold as well as tissue-inducing substances such as growth factors to induce the cells from the native tissue near the region of repair to be mobilised and subsequently populate and proliferate within the prosthesis. One such example of tissue engineering by *in vivo* regeneration of tissue is oesophageal replacement prosthesis first reported by Takimoto *et al.* [9, 11, 49-52].

This new type of artificial oesophageal replacement consists of an outer tube of collagen sponge made from mainly Type I collagen and an inner silicone tube that is reinforced with a nylon mesh made from silicone glue for support. [9, 11, 49-52]. The prosthesis is then surgically implanted into the body to replace a segment of the original oesophagus to allow for regeneration *in vivo*. Figure 10 shows the construction of the new type of artificial oesophagus made from collagen sponge and a nylon reinforced silicone tube.

Figure 10. Artificial oesophagus constructed using silicone tube and collagen sponge (Nakamura and Shimizu, 2000) [9]

It has been reported that a segment of the thoracic oesophagus up to 9 centimetres in length can be regenerated using this method with histological findings showing that the regenerated tissue has stratified epithelia, circular and longitudinal muscle layers as well as oesophageal glands [9, 11, 49-51].

However, it is important to note that *in vivo* tissue regeneration requires the precise control of the rate of scaffold degradation or resorption. As the scaffold loses mechanical strength as it degrades, rapid degradation would cause the cell/scaffold construct to lose its mechanical strength too rapidly, causing it to collapse before tissue regeneration can be completed. Should the scaffold degrade too slowly, residual scaffold material may be left behind and embedded within the regenerated tissue [11] and may even hinder regeneration [10, 17].

In vivo regeneration has also been used to regenerate other tissue types such as nerves [53], skin [10, 53], cartilage and knee meniscus [53].

The second type of tissue engineering, which is *in vitro* reconstruction, requires the three dimensional assembly of cells and scaffolds through the use of signals to form a living and functional tissue outside the body before it is able to be used. There are currently two approaches for *in vitro* tissue regeneration.

In the open system, cells are cultured *in vitro* are seeded onto a scaffold. The cells are then allowed to attach to the scaffold and populate within it for an appropriate amount of time before being transplanted into the body and be incorporated into the host. For the closed system, the cells are also grown onto matrices into three dimensional tissue structures. However, they are isolated from the body through the use of a membrane that allows mass exchange of nutrients and waste but separates the tissue construct from the host to prevent its destruction as a result of immune rejection [11, 14].

The tissue engineering approach via *in vitro* reconstruction offers the advantages of being able to control the growth environment and attempts to provide the optimal conditions required by the cells and scaffold to assemble into a three dimensional tissue. It also allows the use of other growth signals such as growth factors or mechanical stimulation through the use of various types of bioreactors

Currently, Sato *et al.* [9, 11, 49, 54-57] have been attempting to develop an artificial oesophagus using the approach of *in vitro* reconstruction, and normal oesophageal epithelium has been shown histologically [11] to be regenerated both *in vitro* and *in vivo* using this method.

Additionally, there are some examples of success for the use of *in vitro* reconstruction for the generation of tissue engineered replacements such as skin grafts [10, 15, 18] and cartilage grafts [18, 58]. Tissue engineering work on other tissue types using this method includes vascular grafts [59-61], cardiac tissues [62, 63] and also bladder tissue [64].

Thus, before any tissue engineered device or replacements can be assembled, the three cornerstones of tissue engineering, namely the cells, signals and scaffold (extracellular matrix) would have to be available.

2.2.3 Cells

In order for any tissue or organ structure to be tissue engineered, cell sources must be acquired. The types of cell sources which can be obtained and used in tissue engineering are autologous, allogeneic or xenogeneic [11, 15, 18-21]. Ideally, cell sources used for tissue engineering should be non-immunogenic, highly proliferative, easy to harvest and have the ability to be differentiated into a variety of cell types of specialised functions [11].

However, the usage of allogeneic or xenogeneic cell sources may result in host immune response and rejection when implanted into the body [14, 15, 19, 20]. Also, certain fully differentiated cell types such as cardiomyocytes, hepatocytes and pancreatic islets do not proliferate well or retain their differentiated function *in vitro* [11, 16, 18, 20].

Researchers have now turned their attention to cells such as fetal, neonatal, genetically manipulated, and pluripotent cells such as mesenchymal and embryonic stem cells [10, 11, 15, 18, 19, 22]. Mesenchymal stem cells have the advantage of being harvested from the patient and subsequently expanded in culture. After the expansion of these stem cells, they would then be induced to differentiate into their required cell type before being used for repair [10, 18, 19].

In addition, embryonic stem cells can be expanded in an undifferentiated form and be induced to form many different cell types. Although, embryonic stem cells have been induced to form tissues such as pancreatic islets and blood [18], much work remains to be done before the understanding of the control of cell/scaffold differentiation for this type of cells is adequate for use in tissue engineering [10, 18, 19].

Alternatively, genetically manipulated cells could be used to alter matrix synthesis, inhibit immune response as well as the synthesis of certain proteins and increase its proliferation rate [19].

2.2.4 Scaffolds

Scaffolds can be made from naturally occurring materials such as collagen and hydroxyapatite, or from synthetic materials, typically polymers such as polyglycolic acid (PGA), polylactic acid (PLA) or the copolymers of both, poly (lactic-co-glycolic acid) (PLGA) [10, 11, 15, 20, 22-24]. However, there is a new and rapidly emerging scaffold material type using a hybrid of both natural and synthetic materials [14, 22]. They can exist in a variety of forms such as sponge-like sheets, gels or highly complex structures with intricate pores and channels [18].

During cell/scaffold development or regeneration, the scaffold serves as a mechanical support against *in vivo* forces to retain its structure [20] and are designed to degrade gradually. The degradation of the scaffold is to allow the extracellular matrix newly formed by the cells to gradually take over the role of mechanically supporting the tissue structure and also to promote their regeneration [15] before being completely incorporated into the host after implantation [18].

Apart from being a mechanical support for the cells, the scaffold also serves as a three dimensional micro environment to facilitate the growth of cells into tissue structures by providing the required chemical and mechanical signals [23, 65, 66]. As such, scaffolds should be of defined shape and contain complex porous architectural structure reflective of the hierarchical structure [23] or architecture [19] of the tissue structure they are attempting to replicate. They serve as a template and guide and direct the process of tissue regeneration [22, 23] such as cell differentiation and cell function [14]. At the same time, the scaffold can also be used to trap products secreted by the cells such as proteins and growth factors [10, 66].

Scaffolds can also be designed to contain bioactive signals such as cell-adhesion peptides and also release growth factors to induce cell differentiation and growth *in vitro* and cell migration *in vivo* [15, 18, 20, 66, 67].

Ideally, the biomaterial used for the scaffold should be able to selectively interact with specific adhesion and growth factor receptors as expressed by the target cells in the surrounding tissue for the repair of the damaged tissue. The scaffold should be also be able to guide the migration of the target cells into the injury site and stimulate their growth and differentiation and finally, to degrade in response to enzymes released by cells during remodelling of the matrix in tune with the tissue repair process [18].

Scaffolds made of synthetic materials have better physical properties such as strength, degradation time, hydrophobicity and also microstructure [11, 14, 21, 68], which are vital to the effectiveness of the scaffold for use in tissue engineering and also offers consistency between batches. However, scaffolds made of naturally derived materials do have the advantage of being a closer representation to the native environment of the cells [21, 68]. But, they may be difficult to extract and isolate from the natural sources [68] and suffer inconsistency in quality between batches [14].

In addition, acellular xenogeneic scaffolds such as the decellularised small intestinal submucosa developed by Badylak [10, 25-27] and subsequently the decellularised urinary bladder matrix as well as decellularised heart valves [69] are also a source of naturally derived scaffolds. These acellular scaffolds are obtained by the removal of native cells from tissue to yield extracellular matrix. This type of scaffold contains many proteins and signals required by the cells and have been shown to be immunologically acceptable without the use of immunosuppressants [10].

2.2.5 Signals

In the previous section, it has been shown that both biochemical and biomechanical signals are involved in the process of tissue assembly and are important for tissue growth and regeneration both *in vivo* and *in vitro* [65, 70].

In fact, as “dynamic reciprocity” exists between the scaffold and the cells with signals being passed and induced between the scaffold and cell in both directions, the cell responding to biomechanical signals from the extracellular matrix and responding with the appropriate biochemical signals to affect the extracellular matrix [71].

These biochemical and biomechanical signals, provided by both growth factors and the physical structure of the extracellular matrix, regulate the function of cells in particular differentiation, proliferation, migration, and apoptosis [66, 71, 72]. The extracellular matrix in turn is remodelled into functional tissue structures for healthy organ function [71].

In addition to the biochemical and biomechanical forces experienced by the cell within the scaffold and extracellular matrix, external applied forces such as fluid shear, in the case of blood vessels, and mechanical strain, in the case of muscles, also serve as signals for the growth and development of tissue constructs. A more detailed discussion on the effects of mechanical stimulation through the use of bioreactors on cell/scaffold constructs *in vitro* will be presented subsequently.

2.2.6 Vascularisation and metabolic requirements

For a tissue construct to be successfully engineered, solutions for fundamental issues relating to the mass transfer in the form of nutrients, gases and metabolic wastes is required [22]. As the tissue construct grows, the metabolic requirements of the construct would increase while the surface area to mass ratio decreases, thereby limiting the amount of mass transfer available for cells at the core of the tissue construct.

Without the aid of a vascular network within the tissue constructs, it may be difficult to keep the tissue construct feasible *in vitro*. Even if the tissue construct is able to be kept alive without the presence of a vascular network *in vitro*, once removed from the controlled environment and transplant, the host body would require a few days to fully vascularise the tissue construct [23] where the cells within the construct would have oxygen to survive only a few hours before cell necrosis occurs without vascularisation to meet metabolic demands [10]. This would be particularly true for the tissue engineering of larger and more complicated organs such as the kidney, liver and heart [11, 17].

Tissue constructs therefore have to be designed to provide access to nutrient media and to promote the development of adequate vascularisation when implanted into the body [10]. Efforts to improve the mass transfer requirements for tissue constructs such as the modification of the porous structure of the scaffold [68, 73] as well as the fabrication of vessel network are currently being explored [22, 73].

Another possible solution to meet the mass transfer requirements of the tissue construct is the use of hydrodynamic flow control of the medium and to optimise the mass transfer between the construct and the media. Experimental studies demonstrate the effects of such control being able to optimise the mass transfer [29, 74].

For successful development of tissue engineered replacement organs and tissues, there is a requirement for different types of bioreactors to provide an optimal environment for the growth and development of cell/scaffold constructs. These bioreactors must be able to provide a controlled biochemical and biomechanical environment in addition to supplying adequate nutrition.

2.3 Bioreactors

Bioreactors are defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions [29]. They are classically used in industrial fermentation processing, waste treatment, food processing and the production of pharmaceuticals and recombinant proteins [29, 41, 75].

For the purpose of tissue engineering, bioreactors should provide an optimal environment for the development of cell/scaffold constructs into functional tissues *in vitro*. Mass transfer requirements such as the transfer and exchange of nutrients, gases and metabolic waste must be attended to and maintained at the optimal conditions for the growth and development of the tissue construct. Additionally, biochemical and biomechanical signals in the form of growth factors and mechanical stimulation specific to the tissue type in culture should be provided whenever possible to replicate the physiological conditions as closely as possible *in vitro*. The process of replicating the physiological conditions of the native tissue *in vitro* is also known as “biomimicry” [76].

In order to meet the requirements of tissue engineering, many different types of bioreactors have been developed to investigate the optimal conditions or environment for different cell and tissue types. In this section, various methods of tissue culture using different types of bioreactors would be introduced and discussed.

2.3.1 Petri Dishes/Static Flasks

Culture of cells within petri dishes or static flasks is the simplest and most widely used method in tissue engineering [77]. This form of bioreactor requires the manual exchange of culture medium every 3 to 4 days in order to remove metabolic waste produced by the cells and the supply of the more nutrients. The gaseous mass transfer requirements of the cells are met through the exchange of oxygen and carbon dioxide at the medium-air interface through the diffusion mechanism. Due to the ineffectiveness of the gas exchange mechanism, only a monolayer of cells up to a thickness of about 10 microns [77] can be produced using this method. Previous work

attempting to produce three dimensional cell/scaffold constructs using this method have led to low cell density and the presence of a necrotic core [29, 78-80] within the construct, and the partial or complete de-differentiation for some cell types [81]. Because of these limitations, this method of culturing cells should only be considered for the purpose of cell expansion [82].

2.3.2 Spinner Flasks/Mixed Flasks

For cells in static culture, the monolayer of cells is not nutrient-limited [77]. However when thicker cell/scaffold constructs are required, the mechanism of passive diffusion is no longer able to provide sufficient mass exchange between the construct and the medium [29, 77]. Thus, in order to alleviate the problem of insufficient rate of exchange of gases between the cell construct and medium, mixed flasks are used.

The mixed flask shown in Figure 11 (a) is a static flask with the addition of a stirrer. The stirrer to provide convection [29, 80, 81] within the bulk medium to maintain the uniformity of the culture within the flask and prevents localised build up of metabolites at the construct-medium interface and improves the overall mass transfer effectiveness [77]. However, there is turbulent flow over the construct surface as a result of the stirring [77, 80] and the cells are exposed to unintended shear forces which do not replicate the physiological environment of the native cell/scaffold type [77, 80]. Manual medium exchange is also required periodically for this type of bioreactor.

(a) Mixed Flask (b) Rotating Wall Vessel (RWV)
Figure 11. Types of bioreactors used in tissue engineering (Adapted from Martin *et al.*, 2004) [29]

Although cell/scaffold constructs cultured using this method produce better constructs as compared to those cultured in static flasks [80], problems of poor mass transfer within the construct itself and shear stresses as a result of turbulent flow have led to a

construct that is populated with cells nearer to the construct-medium interface and a relatively acellular core [29, 80].

2.3.3 Rotating Wall Vessel

Rotating wall vessels (RWV) are bioreactors that are made out of two concentric cylinders with a medium inlet at one end and an outlet on the central cylinder [83]. They were designed to study the effects of microgravity on the assembly of three dimensional constructs [79, 80, 83, 84]. Figure 11 (b) shows a figure of the rotating wall vessel.

The RWV also provides a convective environment in which the media is mixed by the movement of the rotating wall and the movement of the tissue construct [83]. Unlike the spinner flask, the flow across the constructs within the bioreactor is laminar in nature which results in low shear stress [29, 79, 80, 83]. The low level of shear stresses promotes the aggregation of cells and yet overcomes the conventional diffusional limits for the mass transfer of nutrients and wastes [29, 79, 80].

Cartilage tissues cultured in RWVs are found to have better mechanical properties compared to those cultured in static or mixed flasks [29] whereas cardiac tissue constructs exhibited elongated cells which are able to contract spontaneously and synchronously [29, 74]. Additionally, macroscopic tissue aggregates up to 1 cm in size are found to be without necrotic cores [80].

2.3.4 Direct Perfusion Bioreactors

Direct perfusion bioreactors dispense fresh medium to the cell tissue constructs at a highly controlled rate. This highly controlled perfusion process allows optimal mass transfer between the construct and the tissue for the growth of the cell/scaffold construct. This method of perfusion has been used to maintain highly metabolic cells [29].

When combined with the use of specially designed scaffolds for the transfer of nutrients [68], the limitations of mass transfer at the core of the cell/scaffold construct

can be reduced [29]. Tissue constructs grown using direct perfusion bioreactors have been shown to enhance the growth of tissue constructs.

In addition, this type of bioreactor can be used to study the effects of shear forces on shear sensitive cells such as vascular endothelial cells, or used for mechanical conditioning of the cell layer through the use of hydrodynamic shear forces due to the flowing nutrient media.

2.3.5 Tissue Specific Bioreactors

As our understanding of cellular biology progresses, it becomes more evident that “biomimicry”, or the replication of physiological conditions *in vitro*, is an important factor for successful culture of specific tissues [85]. Conventional bioreactors that have been used for tissue culture may not meet this requirement.

Additionally, as the physiological conditions for different cell types located in different parts of the body are vastly different, different types of biochemical and biomechanical signals are required to grow different tissues. Thus, it is impractical to design a single “universal” bioreactor and attempt to meet all our tissue engineering requirements with a single device.

The ideal bioreactor for tissue engineering would be designed to meet all the mass transfer requirements of the specific tissue type, and also to replicate the physiological conditions normally encountered *in vivo*.

Tissue specific bioreactors have been designed for cartilage [58], vascular grafts [86, 87], cardiac tissue [62, 88-92], bladder tissue [93] and other tissue types.

In order to further improve and optimize and improve the effectiveness of these tissue specific bioreactors, another type of bioreactors incorporating mechanical stimulation has been designed to allow the study of cellular response under different mechanical stimuli. This area of biology is often termed “mechanobiology”.

2.3.6 Mechanical Stimulation Bioreactors

The environment within the body is an extremely complex system [94]. Thus, in order to understand the effects that mechanical forces have on the cells and tissues within the body, mechanical stimulation bioreactors are designed and constructed to facilitate the study of these phenomena.

Mechanical stimulation bioreactors allow the full or partial replication of the mechanical environment found *in vivo* for the intended cell/scaffold type through the control of the *in vitro* environment.

Compressive Loading Systems

There are generally two types of compressive loading systems that are used to study the effects of cells in an *in vitro* environment. Hydrostatic pressurisation, as shown in Figure 12 (a), allows the study of the cells under compressive mechanical forces by subjecting the cells to an increase in hydrostatic pressure through the loading of force to the fluid. This method also allows the use of negative (below zero gauge pressure) as well as positive pressurisation [94].

Compression via the use of hydrostatic pressurisation has the advantage of uniformity of pressure and is able to apply both transient and static loadings. As there is no direct contact with the cells when subjected to compression, the possibility of localised compression is eliminated. As the loading is not transferred through an intermediate medium such as a substrate, the loading is independent of the cell adhesion condition.

However, as hydrostatic pressure affects the solubility of gases in the medium, high levels of partial pressure of oxygen and carbon dioxide may result, which may adversely affect the cellular response. Special preparations of the experimental samples and *in vitro* conditions may be required to yield meaningful results depending on the nature of investigation [70, 94]. Alternatively, the compression can be done in the absence of the gas phase to circumvent problems associated with increase in dissolved gas [70].

(a) Hydrostatic pressurisation

(b) Platen displacement

Figure 12. Compressive loading bioreactor systems (Adapted from Brown, 2000) [94]

Another form of compressive loading known as platen displacement applies compressive forces directly to the cells through the use of compression plates. The cell/scaffold construct is placed between a fixed surface and a mobile plate which provides a predetermined displacement as shown in Figure 12 (b). The loading regime for this type of compressive loading can also be static or cyclic.

However, this form of compressive loading suffers from the “barrelling effect” which causes the cell/scaffold construct to bulge outwards at the centre away from the construct-plate interface. This is caused by frictional forces between the construct and the plate which are required to keep the construct in position. As such, localised compression can be a problem and may influence the result of such a study.

Due to its similarity in concept to *in vivo* conditions of cartilage, this method of mechanical stimulation is used extensively for mechanical conditioning of bio-artificial cartilage constructs [95] and to study the effects of the tissue under different loading conditions [96].

Fluid Shear Systems

Fluid shear systems provide mechanical stimulation to cells via the application of fluid shear stress at the construct-medium interface. Shear sensitive cells are known to exhibit a wide variety of cellular responses when exposed to shear stress [94].

There are generally two principal types of fluid shear systems: Rotating disc/cone, and flow chamber systems.

The rotating disc/cone systems include parallel-disc viscometers and the cone-and-plate viscometers. The parallel-disc viscometer comprises two circular parallel plates with cells and nutrient media located between them. One of the plates rotates at a constant angular velocity to generate laminar flow over the cells to provide fluid shear stresses [70].

The cone-and-plate viscometer consists of a flat disc and a specially profiled cone. Similarly to the parallel-disc viscometer, the cells and nutrient media would be placed on the flat disc and the cone then rotate at a constant angular velocity to generate a laminar flow profile across the cross section of the cone and the flat disc to generate fluid shear stresses. Figure 13 (a) shows the diagram for a cone-and-plate viscometer [70, 94].

In the parallel-plate viscometer, the linear velocity of the fluid flow experienced by the cells is a function of the radius and the shear forces generated along the radius of the parallel-disc viscometer will vary. In the case of the cone-and-plate viscometer, the specially profiled cone ensures that fluid shear stresses experienced by the cells will not vary with the radius [70].

Figure 13. Fluid shear stress bioreactor systems (Adapted from Brown, 2000) [94]

The flow chamber systems consist of two different configurations, the parallel plate flow chamber and the radial flow chamber [70].

The parallel plate flow chamber as shown in Figure 13 (b) generates fluid shear stress by the movement of the medium across the surface of the cell culture. The cell culture undergoing fluid shear stresses are placed along the length of the parallel-plate flow chamber. For the flow of culture medium to occur, a pressure gradient must be present between the inlet and the outlet of the flow chamber. In order to provide continuous fluid shear stress to the cell culture, the culture medium is re-circulated from the outlet back into the inlet. This method provides constant fluid shear stresses along the length of the chamber [70, 94].

The radial flow chamber consists of two parallel circular discs similar to those found in the parallel disc viscometer. The cells to be subjected to fluid shear stresses are placed between the two parallel circular discs. In the case of the radial flow chamber, there is no rotational movement of the discs with respect to each other. Instead, flow is introduced from the centre of the parallel discs and flow outward radially towards the edges of the discs to be collected and subsequently re-circulated into the inlet of the radial flow chamber [70].

The fluid shear stress generated using the radial flow chamber is the maximum at the centre of the chamber and decreases with the increase of radial distance until the edge. As such, it not possible to generate uniform shear stresses throughout the chamber using this method. However, it is possible to observe the effects of a range of shear stresses within a single experiment [70].

Tensile Loading Systems

Tensile loading systems, also known as mechanical stretching systems, apply tensile forces to cells through substrates. Tensile loading on the cells is usually reported as strain. The substrate is usually a flexible elastomeric material such as silicone rubber (Silastic®) [94, 97-101] or polyurethane [94, 102, 103]. Textile scaffolds and decellularised extra cellular matrices may also serve as the scaffold [104].

However, the effectiveness of this form of tensile loading depends on the adhesion condition between the cells and the substrate or scaffold. If the cells do not fully adhere to the scaffold, then the cells will not be subjected to the same strain as the scaffold resulting in experimental error [70]. There are two variations of tensile

loading: uniaxial (longitudinal) loading and biaxial loading as shown in Figure 14 (a), (b) and (c), (d) respectively [70, 94].

Figure 14. Tensile loading bioreactor systems (Adapted from Brown, 2000) [94]

Uniaxial strain or longitudinal strain is the application of strain along one axis and biaxial strain is the application of strain along two perpendicular axes. These substrates are usually connected to a pre-programmed actuation device which is able to perform either static tensile loading or cyclic tensile loading.

Parameters which can be varied using the tensile loading bioreactor systems are its strain magnitude, frequency and duty cycle.

For uniaxial tensile loading, the cells on the substrate may be subjected to tensile loading via the use of grip displacement [104, 105] as shown in Figure 14 (a) or bending [106] as shown in Figure 14 (b). Figure 15 shows a typical uniaxial tensile loading bioreactor system using grip displacement [105].

Figure 15. Uniaxial strain tensile loading bioreactor system using grip displacement [105]

Biaxial tensile loading can be performed using two methods: out-of-plane circular substrate distension; and in plane substrate distension. Figure 14 (c) shows biaxial tensile loading via out-of-plane circular substrate distension, while Figure 14 (d) shows the in-plane substrate distension.

Out-of-plane circular substrate distension utilises a circular substrate that is clamped at its periphery. Thus, for biaxial tensile loading to occur, the substrate must be distended out of its original plane either upwards or downwards. Various mechanisms are used to achieve out-of-plane distension.

Platen displacement uses a specially profiled plate to apply displacement to the substrate. For the prong displacement, a circular plate is used to displace the substrate along its central axis. Using vacuum, air is removed from the lower chamber of the biaxial strain tensile loading system so that the substrate is distended downwards. For the fluid displacement method, fluid is allowed to enter the lower chamber of the biaxial strain tensile loading system to distend the substrate upwards.

For biaxial tensile loading using out-of-plane substrate distensions, it is important to note that the strain field in the substrate is not equi-biaxial throughout. This is a result of the circular substrate being clamped at the periphery. However, constant radial strain can be achieved with the tangential strain being maximum at the centre of the of the substrate and decreasing along the radial direction until it reaches zero at the periphery [29, 102].

In order to achieve equi-biaxial tensile loading, in-plane substrate distension method would have to be utilised [100, 101, 103]. In-plane substrate distension supplies biaxial tensile loading to the substrate without the substrate having to be distended out of the original plane. In-plane substrate distension can be achieved using the following methods.

Frictionless platen displacement uses circular rings to distend the surface, thereby providing equivalent amount of distension in all directions. Using vacuum with a frictionless platen supporting the cells in the substrate, the vacuum generates in-plane substrate distension on top of the frictionless platen via the downwards distension of the surrounding substrate. Finally, biaxial traction mechanically distends a flat substrate is using two set of grips and displaced in perpendicular directions as shown in Figure 14 (d).

For in-plane substrate distension, the equi-biaxial tensile loading is only valid should the frictional forces involved in the contact surfaces are frictionless or close to frictionless. Additionally boundary effects must be negligible in the central regions for equi-biaxial strain to be valid [94].

2.4 Effects of Mechanical Forces on Cells

All cells and tissue *in vivo* are constantly subjected to mechanical forces. These mechanical forces provide vital signals to regulate cellular functions such as differentiation, proliferation, migration and apoptosis [66, 71, 72].

Increases in cell proliferation rate and changes in tissue morphology such as phenotype expression [107] and cellular alignment/orientation under the influence of mechanical strain have been observed by researchers [30-33]. Apoptosis is also observed and can be controlled through the use of mechanical stretch [108, 109].

Additionally, the dynamic reciprocity of biochemical and biomechanical signals between the cells and the extracellular matrix regulates cell functions as well as the remodelling of the extracellular environment [71].

Increasing evidence suggests that mechanical forces, already known to be important modulators of cell physiology, may have higher levels of control over cellular functions.

Also, when mechanical forces simulating physiological conditions are applied on cell/scaffold constructs, the resulting engineered tissues have shown to improve the structural and function properties [28, 29, 110-113].

Previously research on vascular cells have shown that hemodynamic forces such as shear force and circumferential strain, as a result of pulsatile forces, have been experimentally proven to be important modulators of vascular cell function and morphology. As a result of mechanical stimulation, mechanosensors such as integrins [72, 114], mechanically activated ion channels (MAC) [70, 114], platelet derived growth factor (PDGF) receptors [115] as well as G (GTP-binding) proteins [70, 114, 116], convert these external mechanical forces into chemical signals which are sent into the cell [70, 72, 117].

This results in secondary messengers such as mitogen-activated protein kinases (MAPK) [118, 119], protein kinase C (PKC) [120], and Akt [121] being activated,

leading to an increase in activity of transcription factors such as activator proteins (AP)-1, AP2, cyclic AMP-responsive element (CRE) [120], early growth response (Egr)-1 and nuclear factor (NF)- κ B. These factors then bind to the DNA which in turn activates a large number of genes that regulate cell function such as cell proliferation, apoptosis, differentiation, morphology, migration and secretory functions.

This mechanism whereby vascular cells sense and transduce *in vivo* mechanical forces into the cell nucleus has only recently begun to be understood [117]. A summary of the process of the mechanotransduction of external mechanical stimuli to the actual cellular response is summarised in the flow chart found in Appendix A.

Apart from vascular cells, isostatic mechanical stretching of rat myometrial smooth muscle cells (SMCs) have also led to an increase in the regulation of the *c-fos* messengerRNA [122, 123]. The expression of *c-fos* mRNA is also observed when rat cardiomyocytes, cardiac fibroblast, endothelial cells and vascular SMCs are mechanically stretched along with other proto-oncogenes such as *c-jun* and *fra-1* [70, 114, 117, 124].

Also, along with the increase in *c-fos* mRNA transcription, increase in mitogens including basic FGF (fibroblast growth factor) [125] and PDGF [115] are also observed when smooth muscle cells are mechanically stimulated or injured.

Although the effects of mechanical stimulation on smooth muscle cells have been extensively studied for vascular cells, few studies have been made by researchers on oesophageal cells [34].

Recent work conducted by Cha *et al.* [126, 127] have studied the effects of 1 dimensional mechanical strain duty cycle on rabbit oesophageal smooth muscle cells grown on three-dimensional polyurethane scaffolds grafted with Type I Atellocollagen. These studies demonstrated the existence of an optimal mechanical strain duty cycle regime for cell-scaffold constructs for both cellular alignment and cellular differentiation through the up-regulation in expression of smooth muscle α -actin [126]. In addition, improvements in the mechanical properties of the strained cell-scaffold constructs have also been reported [127].

For this project, investigations on the effects of 2-dimensional mechanical stimulation on oesophageal smooth muscle cells would be conducted in an effort to better understand the cellular process of proliferation and differentiation for application in the tissue engineering of the oesophagus.

3 METHODOLOGY

To facilitate the study of the effects of two dimensional mechanical strain on oesophageal smooth muscle cells, a biaxial tensile loading bioreactor system is first developed. In addition, a suitable actuation membrane/scaffold that is both biocompatible and non-cytotoxic is developed. Finally, a primary cell line of oesophageal smooth muscle is isolated from porcine oesophageal tissue.

Figure 16 below shows the relationship between the bioreactor system, actuation membrane/scaffold and the primary cells developed and used in this study.

Figure 16. Relationship of bioreactor system, actuation membrane/scaffold and isolated cells used in study

3.1 Design of Two-Dimensional Strain Bioreactor System

3.1.1 Cell Culture Environment

To study the effects of two dimensional mechanical strain on oesophageal cells, a bioreactor was designed based on the conceptual design for biaxial strain loading by out-of-plane circular substrate distension using a vacuum as found in Gooch and Tennant [70]. By using the out-of-plane circular substrate distension concept for applying biaxial strain, we are able to investigate the effects of varying amounts of tangential strain on cell orientation/alignment. In addition, the advantages behind this design are the convenience for experimental usage and mechanical reliability.

However, to achieve consistent and predictable displacement, actuation mechanism was changed from a vacuum to a hydraulic system. The incompressible nature of the liquid allows the displacement of the bioreactor membrane (or substrate) to be easily calculated from the volume changes so as to derive the resultant strain that is transferred to the cell/scaffold construct through the substrate.

As such, a conceptual design of the two-dimensional strain bioreactor was finalised, shown schematically in Figure 17. The conceptual design comprises an upper chamber used for cell culture, and a lower chamber which is used to apply hydraulic pressure. In between the chambers, a sheet of membrane used to separate the actuating fluid from the nutrient media. Cells are cultured on the upper surface of this membrane, which flexes in response to the pressure difference ($P - P_{\text{atm}}$).

Figure 17. Schematic of conceptual design of two-dimensional strain bioreactor

For the purpose of this project, two prototypes of the two-dimensional strain bioreactor were designed. A single well prototype of the two-dimensional bioreactor is designed to be used for the investigation of its working principles as well as the state of strain on the bioreactor membrane, while a second six-well bioreactor was designed for the actual study on of the effects of two-dimensional strain mechanical stimulation on oesophageal cells.

Based on the conceptual design, a detailed design of the single well prototype was finalised. The bioreactor is of a simple design consisting of two polycarbonate blocks as the main housing. Two O-rings are used as seals to prevent culture medium from leaking out of the bioreactor and contamination from outside getting inside the cell culture chamber. Additionally, the O-rings also serve as a mounting position for the bioreactor membrane. The bioreactor is held together by 8 screws.

For the bioreactor, the materials used for its fabrication must be biocompatible and must not be cytotoxic. It must be able to be subjected to temperatures of 121°C during steam sterilisation or autoclaving. It must also be chemically resistant to common agents used for sterilisation such as ethanol. Machining grade polycarbonate was selected as the material used for the fabrication of the bioreactor. The O-rings are in close proximity to the cell culture environment, and must therefore be biocompatible. Silicone rubber O-rings are selected to be used in the bioreactor. The screws used for fastening were M3 x 50mm socket head cap screws made of 316L stainless steel. A vinyl sheet was used as the membrane during experiments to characterise the deflected profile and strain.

Figure 18 shows the picture of the single well bioreactor prototype together with a syringe used for hydraulic actuation. The drawings for the detail design of the bioreactor can be found in Appendix B.

Figure 18. Fabricated single well prototype of the two-dimensional strain bioreactor with actuation syringe

For the purpose of the *in vitro* studies of the effects of mechanical strain on oesophageal smooth muscle cells, a six-well variant of the two-dimensional strain bioreactor was also designed and fabricated based on the same operating principles as the initial design.

The major differences in design from the first prototype are the introduction of multi wells within the same bioreactor and the reduction of the well diameter from 50mm to 35mm. The reduction in well diameter is made to allow ease of comparison of results between the mechanically strained cells and those grown on standard six-well tissue culture polystyrene microplates during *in vitro* studies.

Figure 19 shows the picture of the fabricated six-well bioreactor. The drawings for the detail design of the six-well variant of the bioreactor can be found in Appendix C.

Figure 19. Fabricated six-well variant of the two-dimensional strain bioreactor

3.1.2 Control System and Linear Actuator

In order to have a completely functional bioreactor, a complete bioreactor system was designed based on the conceptual approach as shown in Figure 20.

Figure 20. Schematic of the complete bioreactor system

The actuation system is controlled by a programmable microprocessor. A syringe containing water as the actuation fluid is attached to the actuator to provide fluid displacement into and out of the lower chamber of the bioreactor. The actuation fluid flowing into the lower chamber of the bioreactor then displaces the actuation membrane to give rise to a change in maximum deflection along the central axis of the bioreactor well. This deflection would then be transferred to the cell/scaffold construct located within the cell culture (upper) chamber. Figure 21 shows the complete bioreactor system with a six-well variant of the two-dimensional strain bioreactor.

Figure 21. Complete bioreactor system with six-well variant of the two-dimensional strain bioreactor

The following sections describe the components of the bioreactor control system and the actuator in detail.

3.1.2.1 Programmable Microprocessor and Control Circuitry

BASIC Stamp 2 (BS2)

A BASIC Stamp 2 (Parallax Inc., Rocklin, CA) programmable microprocessor is used to control the linear actuator. The use of a microprocessor instead of a “hard-wired”

electronic circuit such as a relay circuit allows the instructional routine to be modified rapidly when required. This allows the two-dimensional strain bioreactor to provide different levels of amplitude and frequency to the cell/scaffold construct within the cell culture chamber of the bioreactor.

The BASIC Stamp 2 microprocessor is a 24 pin DIP (Dual Inline Package) module which is able to control and monitor switches, timers, motors and sensors. It can be programmed using the PBASIC language.

Features of the BASIC Stamp 2 microprocessor are as follows:

- Processor clock speed of 20 MHz
- Program execution speed of about 4000 instructions/sec
- RAM size of 32 bytes (6 bytes for I/O and 26 bytes for variables)
- EEPROM size of 2048 bytes (about 500 instructions)
- 16 I/O pins and 2 synchronous serial pins

The BS2 microprocessor requires a voltage input of between 5 to 15 volts DC and is able to provide (source) 20 mA and absorb (sink) 25 mA at each of the 16 I/O pins up to a total of 40 mA and 50 mA as source and sink respectively.

Instructional routines can be programmed using the BASIC Stamp Windows Editor provided by Parallax Inc. on PCs running Microsoft® Windows Operating Systems and sent to the BS2 microprocessor via the RS-232 serial port.

Control Circuitry

Although the BASIC Stamp 2 microprocessor is able to provide current from the I/O pins, the maximum current provided is insufficient for the proper operation of the DC motor used in the actuator. Thus, to utilise the BS2 microprocessor for the control of the actuator, a control circuitry is designed around the microprocessor.

The control circuitry for the BS2 microprocessor operates the L298N Dual Full-Bridge driver (ST Microelectronics, France) to control the speed and direction of the

DC motor. For the case of driving the DC motor, only half of the L298N circuitry is utilised, therefore the L298N is used as a half-bridge driver.

The half bridge driver is controlled using the BASIC Stamp 2 microprocessor. The BS2 microprocessor is programmed using the PBASIC language with instructional routines to provide logic signals to control the power supplied to the DC motor as well as the direction and velocity of movement of the horizontal slider mechanism. Two limit switches control the stroke length of the linear actuator.

Figure 22 shows the schematic diagram of the control circuitry used to control the actuator.

Figure 22. Schematic diagram of the control circuitry of the actuator using the BASIC Stamp 2 microprocessor

A working prototype of the control circuitry with the BASIC Stamp 2 microprocessor is shown in Figure 23.

Figure 23. Control circuitry incorporating the BASIC Stamp 2 microprocessor

To allow the actuator to provide cyclic strain to the cell/scaffold construct, the BASIC Stamp 2 microprocessor is programmed to control the actuator to provide the cyclic volumetric displacement profile as show in Figure 24.

Figure 24. Cyclic volumetric displacement profile of the Actuator

DC Motor

The motor used for the actuator is a Crouzet Automatismes 82 802 0-12V-16rpm DC Geared Motor. The motor is a 12 Volt DC brushed motor connecting to a gearbox with a gear ratio of 162.5:1 and an output rotational speed of 16 rpm. The output torque is between 1.2 to 2 Nm with a maximum power output of 17 Watts.

To control the direction of rotation, the direction of the direct current supplied to the DC motor has to be bidirectional. The BASIC Stamp 2 microprocessor controlled circuitry through the use of the L298N half-bridge driver is able to provide bidirectional DC power to the motor.

Power Supply

To provide the power for the BASIC Stamp 2 microprocessor, the control circuitry and the DC motor, the Mean Well D-50 Switching Power Supply is used. The Mean Well D-50 Switching Power Supply is a regulated power supply capable of providing up to 50 Watts of power via a +5 Volt terminal and a +12 Volt terminal. The +5 Volt terminal is able to provide a maximum current of 6 Amperes while the +12 Volt terminal is able to provide a maximum current of 2 Amperes.

For the case of the actuation system, the + 5 Volt terminal would be connected to the BASIC Stamp 2 microprocessor while the +12 Volt terminal would be connected to the L298N half-bridge driver to supply bidirectional DC power to the motor.

The specification sheets for the BASIC Stamp 2 microprocessor, ST Microelectronics L298N Dual Full-Bridge driver, Crouzet Automatismes 82 802 0-12V-16rpm DC Geared Motor and the Mean Well D-50 Switching Power Supply can be found in Appendix D.

Also, the source code of the PBASIC programme used to control the linear actuator can be found in Appendix E.

3.1.2.2 Actuator

The actuator consists of a high torque DC motor (Crouzet Automatismes, France) connected to a timing belt to drive a horizontal slider mechanism. Two limit switches are located along the path of the horizontal slider mechanism to control the stroke length of the actuator. These toggle switches are connected to the BASIC Stamp 2 microprocessor controller circuitry to serve as sensors. Upon contact with the toggle switch, the limit switch sends a pulse to the BS2 microprocessor to reverse the direction of travel by the slider mechanism.

To provide fluid displacement into and out of the lower chamber of the bioreactor, the syringe is placed on the syringe fixture located on the actuator. The plunger is then connected to the horizontal slider mechanism to be driven by the DC motor. Figure 25 shows the actuator with the syringe attached to the horizontal slider mechanism.

Figure 25. Actuator with syringe

3.1.3 Bioreactor Actuation Membrane

For the two-dimensional strain bioreactor to be able to impart mechanical strain on oesophageal cells, a suitable bioreactor actuation membrane must be obtained. The bioreactor actuation membrane should be a flexible membrane with mechanical properties such as high tensile strength, able to endure large strains and be fatigue resistant. In addition, the bioreactor actuation membrane must also be biocompatible, non-cytotoxic and be able to support cell attachment.

In view of the above-mentioned material property requirements for the bioreactor actuation membrane, Chronoflex® AL-80A (Cardiotech International, Inc., Wilmington, Massachusetts) is selected [105]. Chronoflex® AL-80A is a medical grade biodurable polyurethane elastomer. It has a hardness of 80 Shore A and has an ultimate elongation of 585%. In addition, it is ether-free and is resistant to the

formation of microcracks in a biological environment which can lead to material failure.

As the Chronoflex® AL-80A polyurethane is obtained in the form of pellets, the polyurethane pellets would have to be processed in order to fabricate the bioreactor actuation membrane.

To fabricate the bioreactor actuation membrane from the Chronoflex® AL-80A pellets, the solvent casting method is used.

First, the Chronoflex® AL-80A pellets are dissolved and homogenised in analytical grade 1,4-Dioxane (Merck, Darmstadt, Germany). The polyurethane-dioxane solution is then casted onto a 280mm by 190mm glass mould with a wet thickness of 2mm. The glass mould with the polyurethane solution is then covered and left to dry in an enclosed fume cupboard for 7 days. Figure 26 shows the polyurethane actuation membrane fabrication in its various stages.

Figure 26. Bioreactor actuation membrane fabrication

After 7 days, the solvent should have evaporated, leaving behind a clear polyurethane membrane. As 1,4-Dioxane is an organic solvent which is cytotoxic, the solvent casted polyurethane membrane is then placed in a vacuum oven for 7 days at room

temperature to evacuate the remaining solvent still present in the polyurethane membrane.

To evaluate the amount of residual solvent still present after fabrication, a sample of the processed polyurethane membrane was then analysed in a Thermo Gravimetric Analyzer, TGA 7 (PerkinElmer, Wellesley, MA). The TGA analysis is performed at the heating rate of 10°C per minute from room temperature to 500°C.

The results of the Thermo Gravimetric Analysis are presented and discussed in the Section 4.1.1.

3.1.4 Theoretical Analyses of Bioreactor Actuation Membrane

Before the bioreactor can yield any meaningful results, the biaxial strain field generated by the bioreactor actuation membrane must be investigated and characterised. For the purpose of this project, the bioreactor actuation membrane was both theoretically analysed and experimentally investigated.

For the theoretical analysis of the bioreactor actuation membrane characteristics, three different approaches were used: classical bending theory, the principle of virtual work/displacement and the geometrical modelling of a spherical section in an attempt to achieve an analytical solution to describe the profile of the bioreactor membrane under varying deflections.

The theoretical analyses of bioreactor actuation membrane are presented and discussed in Section 4.1.2.

3.1.5 Experimental Investigation of Bioreactor Actuation Membrane

In order to obtain an analytical solution for the state of strain of the bioreactor actuation membrane using the geometrical modelling of a spherical section, an experimental investigation of the deflection profile of the bioreactor membrane was conducted.

For the investigation of the deflected profile of the bioreactor actuation membrane, the Mahr OMS 400 Coordinate Measuring Machine (CMM) (Mahr Federal Inc., Providence, RI) was used. In this experiment, a laser sensor was used to determine the deflected shape of the membrane due to the change in volume.

Before any measurement can begin, the CMM must be calibrated. The X-Y plane of the CMM was set using a series of calibration points on the rim of the bioreactor well. To obtain the well centre, a second group of calibration points were taken along the edge of the bioreactor well. After the calibration of the X-Y plane as well as locating the well centre, the coordinates are then offset from the X-Y plane with the centre of the well on the X-Y plane taken as the origin.

So, in order to characterise the deflected profile of the bioreactor actuation membrane, a line scan was performed along the radial direction from the well centre to the rim of the well. The line scan was performed in 4 different radial directions to check for consistency and each set of line scans was repeated 3 times for consistency. A total of 5 sets of deflection data, each with different maximum deflection at the well centre, were measured experimentally and plotted to obtain the bioreactor membrane deflection profile.

The results obtained experimentally using the above method will be presented and discussed in the Section 4.1.3.

3.2 Establishment and Characterisation of Primary Oesophageal Smooth Muscle Cells

For the purpose of this research, the porcine model was used for *in vitro* studies conducted in this project. Fresh porcine oesophagus samples were obtained from a local abattoir. Oesophageal smooth muscle cells (SMCs) were then isolated from the oesophagus samples and expanded *in vitro* inside a humidified 5% CO₂ incubator at 37°C (Sanyo Electric Co., Ltd., Japan).

3.2.1 Isolation of Primary Oesophageal Smooth Muscle Cells

To isolate primary oesophageal smooth muscle cells from porcine tissue, oesophageal samples were collected from the abattoir. The oesophageal sample extracted from the pig was about 20mm in length, from a position about 10mm above the lower oesophageal sphincter. The extracted sample is then immediately placed in the transport buffered solution and placed in packed ice for transfer to the laboratory.

Upon reaching the laboratory, the porcine oesophageal sample was transferred into a Biological Safety Cabinet Class II (Pall Gelman Sciences Corporation, Ann Arbor, Michigan) laminar flow hood and to be further processed. The tubular sample was removed from the transport buffered solution then cut longitudinally to form a sheet. The mucosa and submucosa layer is then removed from the sample and discarded, leaving behind the muscularis externa. The muscularis externa is then further rinsed twice in fresh transport buffered solution before being minced into cubes of about 1 to 2mm³.

The minced samples are then placed into a 50ml centrifuge tube along with 5ml of enzymatic digestion solution and incubated in 37°C for 1 hour. After 1 hour, 2mg of Elastase Type III was added directly into the enzymatic solution and further incubated for an additional hour. After 2 hours of incubation, the enzymatic digestion solution was sieved through a 100µm cell strainer (BD Biosciences, Franklin Lakes, NJ) and collected into a sterile container to give a single cell suspension solution. The single cell suspension solution was then centrifuged and the supernatant discarded. The cell pellet is then resuspended in 1 ml of primary culture medium and seeded onto 60mm

diameter tissue culture plates (Nunc A/S, Roskilde, Denmark) and grown to confluence inside a humidified 5% CO₂ incubator at 37°C. Fresh primary culture medium was exchanged with exhausted culture medium every 2 to 3 days.

A detailed protocol used for the isolation of primary oesophageal smooth muscle cells can be found in Appendix F together with the formulations of the solutions used in this procedure.

3.2.2 Sub-cultivation of Primary Oesophageal Smooth Muscle Cells

After about 2 to 3 weeks, the oesophageal smooth muscle cells isolated in accordance to the methods described in Section 3.2.1 would have grown to form confluent monolayer. When cells within a tissue culture container have grown to confluence, the cell growth rate will start to decline or cease completely [128]. To continue cell proliferation the cells would be required to be detached from the existing tissue culture container and be divided and transferred into new tissue culture containers. The process of transferring confluent cells from an existing tissue culture container and divide them among new containers is called sub-culturing or passaging. After the first sub-culture, the isolated primary oesophageal smooth muscle cells are now considered a primary cell line.

To sub-culture anchorage dependence cells, as in the case of the oesophageal smooth muscle cells isolated previously, exhausted primary culture medium is first removed. The confluent monolayer of cells are then rinsed twice with sterile 1× PBS and aspirated to remove any cell debris. To dissociate the confluent monolayer from the existing tissue culture container, 0.25% Trypsin-EDTA solution (Sigma, St. Louis, Missouri) is added to the tissue culture container and incubated for 3 minutes at 37°C. After incubation, an equal amount of cell culture medium is added to neutralise the Trypsin.

The cell suspension within the tissue culture plate is then aspirated and transferred into a sterile centrifuge tube and centrifuged to form a cell pellet. The supernatant is then discarded and the cell pellet is then resuspended in cell culture medium. The

resuspended cells are then transferred into new tissue culture containers at the ratio of 1:5 and incubated in a humidified 5% CO₂ incubator at 37°C to attach and proliferate.

A detailed protocol used for the sub-cultivation of the porcine oesophageal smooth muscle cells can be found in Appendix G together with the formulations of the solutions used in this procedure.

3.2.3 Characterisation of Primary Oesophageal Smooth Muscle Cells

In order to ascertain the cell type of the isolated from the porcine oesophageal sample, immunocytochemical staining is performed. The isolated cells were stained with mouse monoclonal primary antibodies for smooth muscle α -actin (Dako, Glostrup, Denmark), desmin (Chemicon International, Inc., Temecula, CA) and smooth muscle myosin heavy chain (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) which are known markers for smooth muscle cells [129-132].

Single cells isolated from the porcine oesophageal samples were seeded onto glass cover slips (Paul Marienfeld GmbH & Co. KG, Germany) coated with rat tail Collagen Type I (BD Biosciences, Franklin Lakes, NJ). The glass slides are then incubated overnight inside a humidified 5% CO₂ incubator at 37°C for the cells to attach and spread. An additional glass cover slip is prepared for each antibody to serve as a negative control during the immunocytochemical staining.

After the cells are attached, they are first fixed using cold methanol at -20°C for 20 minutes and rinsed with 1× PBS. Blocking medium is then dispensed onto the glass cover slip and incubated for 2 hours at room temperature to block out non-specific binding sites present within the cell cytoplasm.

After 2 hours of blocking, the blocking medium is removed and replaced with the primary antibody solution. The primary antibody solution is then incubated for another 2 hours at room temperature. For the negative controls, Negative Control mouse IgG1 (Dako, Glostrup, Denmark) solution is used in place of the respective primary antibodies.

In order to allow the location of the cytoskeletal proteins within the cell to be observed through fluorescence microscopy, a secondary antibody is required to link the primary antibody to a third antibody, which is also called the detection system. The secondary antibody to be used to link the primary antibody to the detection system is Biotinylated Polyclonal Goat anti-Mouse Immunoglobulins (Dako, Glostrup, Denmark). The secondary antibody solution is dispensed onto the glass cover slip and incubated for 2 hours at room temperature to react and bind with the monoclonal mouse primary antibody.

Finally, the third antibody which is used as the detection antibody is Streptavidin TEXAS RED® conjugate (Oncogene Research Products, La Jolla, CA) for red fluorescence or Streptavidin FITC conjugate (Dako, Glostrup, Denmark) for green fluorescence. The tertiary antibody solution is dispensed onto the glass cover slip and incubated for 2 hours at room temperature in the dark to minimise photobleaching of the fluorescence dye. The streptavidin would react and bind with the biotin to show the location of the primary antibody.

To observe the cell nucleus along with the cytoskeletal proteins, the cells are stained with DAPI (Sigma, St. Louis, Missouri) solution for one minute. The glass cover slips are then mounted onto glass slides with Dako® Fluorescent Mounting Medium (Dako, Glostrup, Denmark) to preserve the fluorescence dye.

The glass slides are then examined using a Zeiss Axioskop 2 Mot Plus (Carl Zeiss Inc., Germany) fluorescence microscope.

A detailed protocol used for the immunocytochemical staining of the porcine oesophageal smooth muscle cells can be found in Appendix H together with the formulations of the solutions used in this procedure.

The results of the immunocytochemical staining are presented and discussed in Section 4.2.

3.3 Evaluation of Cytocompatibility of Bioreactor Actuation Membrane

3.3.1 Cell Proliferation Test

To evaluate the biological suitability of the solvent casted Chronoflex® AL-80A polyurethane membrane for use as the bioreactor actuation membrane, a cell proliferation test was conducted on the bioreactor actuation membrane using it as a 2 dimensional scaffold to support cell growth.

As the surface of the polyurethane membrane is hydrophobic, oxygen plasma is used to reduce the surface hydrophobicity by modifying the surface chemistry of the membrane. Oxygen plasma is applied using a Series 800-II Micro-RIE Reactive Ion Etcher from Technics (Japan). Porcine oesophageal smooth muscle cells were used for the cell proliferation test and isolated in accordance to the method described in Section 3.2.1.

The oxygen plasma treated polyurethane membrane is first cut into discs of 35mm in diameter. Disinfection of the polyurethane discs is carried out with 70% ethanol and washed with sterile 1× phosphate buffered saline (1× PBS) (Sigma, St. Louis, Missouri) before being placed into six-well tissue culture treated microplate (NUNC, Denmark).

Passage 6 porcine oesophageal smooth muscle cells (SMCs) are then seeded onto polyurethane discs at the cell density of approximately 5200 cells per cm². The microplate wells were then filled with 2 ml of cell culture medium (as described in Appendix G) and then incubated in a 5% CO₂ humidified incubator (Sanyo Electric Co., Ltd., Japan) at 37°C.

The exhausted cell culture medium is then removed every 2 days and the cells grown on the polyurethane membrane is rinsed in 1× PBS. Fresh cell culture medium is then added before being placed back into the incubator. The porcine oesophageal smooth muscle cells are then allowed to grow on the polyurethane membrane for up to 14 days.

The cell number was determined for days 3, 7 and 14. To determine the cell number, the cells were first detached from the polyurethane membrane using $1\times$ Trypsin-EDTA solution (Sigma, St. Louis, Missouri) and centrifuged to obtain a cell pellet. The cell was then resuspended in 100 μ l of fresh complete medium and diluted accordingly with 0.4% Trypan Blue solution (Sigma, St. Louis, Missouri) before being counted using a Neubauer improved bright-line haemocytometer (Paul Marienfeld GmbH & Co. KG, Germany). The cell counts were performed in triplicates.

The results of the cell proliferation test are presented and discussed in the Section 4.3.1.

3.3.2 Cell Attachment Test

In order to study the effects of two-dimensional strain mechanical stimulation on oesophageal smooth muscle cells, the quality of cell attachment to the substrate would be an important factor to ensure that the mechanical strain provided by the bioreactor actuation membrane would be imparted to the oesophageal smooth muscle cells.

As such, a cell attachment test is conducted on the oxygen plasma treated polyurethane membrane and compared with 3 other surfaces, namely, tissue culture polystyrene (TCPS), Gelatin coated tissue culture polystyrene (GELATIN), and Collagen Type I coated polystyrene (COL I).

The experimental procedures used for the cell attachment test is identical to that as described in Section 3.3.1 for the cell proliferation test with the exception that the cells that are seeded onto the substrates are only incubated overnight and detached the next day to be counted.

The results of the cell attachment test are presented and discussed in the Section 4.3.2.

3.4 Two-Dimensional Mechanical Strain Stimulation of Oesophageal Smooth Muscle Cells

The main function of the oesophagus *in vivo* is to provide peristaltic motion to transport food from the mouth, into the stomach during ingestion. As such, the underlying smooth muscle cells found within the oesophagus would routinely experience mechanical forces as a result of such motion.

So, in order for the study of the effects of two-dimensional mechanical strain stimulation on oesophageal smooth muscle cells to be meaningful, a suitable mechanical strain regime that closely resembles what the cells experience *in vivo* would be required. As such, a mechanical strain cycle based on the period of the pressure waveform generated by the oesophagus during ingestion of solid food would be investigated [37, 105].

The oesophageal smooth muscle cells would be stimulated at 5 cycles per minute for 420 cycles a day. The duty cycle of the mechanical strain cycle chosen for the investigation is 50%. Figure 27 shows the mechanical strain cycle used to mechanically stimulate the oesophageal smooth muscle cells *in vitro*.

Figure 27. Mechanical strain cycle for oesophageal smooth muscle cells

For the purpose of this project, the radial strains that would be investigated using this two-dimensional mechanical strain model would be 0% (static control), 2% and 5%. The corresponding maximum deflection ($y_{\max}$) for 2% and 5% radial in the bioreactor

membrane would be 3.1mm and 4.85 mm respectively and is within the theoretical limit of the geometrical model.

3.5 Assessment of Cell Response to Two-Dimensional Mechanical Strain Stimulation

To ascertain the effects of two-dimensional mechanical strain, the mechanically stimulated cells would be compared with the static controls and assessed using the following methods listed in this section.

3.5.1 Cell Alignment

Alignment of the cells grown on the bioreactor actuation membrane/scaffold would be observed using phase contrast microscopy. Three areas of the bioreactor actuation membrane/scaffold would be examined, namely the well edge, mid-radius and well centre.

3.5.2 Cell Proliferation

To determine the effects of two-dimensional mechanical strain have on the proliferation of oesophageal smooth muscle cells, a cell proliferation assay is performed using the CellTiter96® AQueous One Solution Reagent (Promega, Madison, Wisconsin) is used. The MTS tetrazolium compound within the reagent is biologically reduced by metabolically active cells into a coloured formazan product that is soluble in cell culture medium. It is suggested that the mechanism for this conversion caused by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells [133].

The cell proliferation assay solution is made by mixing 1 part of CellTiter96® AQueous One Solution to 4 parts of cell culture medium. To start the cell proliferation assay, the exhausted cell culture medium is first discarded and the assay solution is added onto the cells. The cells are then incubated in a humidified 5% CO₂ incubator at 37°C for 75 minutes. After completion of incubation, the assay solution is then removed from the cells and dispensed into a 96 well microplate (Nunc A/S, Roskilde, Denmark) and measured for their light absorbance at 490nm using a GENios microplate reader (TECAN Austria GmbH, Salzburg, Austria).

3.5.3 Cytoskeletal protein analysis

As the purpose of stimulating the oesophageal smooth muscle cells through the use of two-dimensional mechanical strain is to mimic the physiological forces experienced by the cells *in vivo*, it is hoped that such biomimicry would allow the cells to revert to its *in vivo* state before being isolated from the oesophagus and achieve differentiation.

Since the phenotype of smooth muscle cells can be determined through the expression of certain specific cytoskeletal proteins, the expression levels of these cytoskeletal proteins can then be investigated. The cytoskeletal proteins that are investigated in this project are smooth muscle α -actin, vimentin, desmin, calponin 1 and smooth muscle myosin heavy chain.

Before the expression of cytoskeletal proteins can be investigated, the oesophageal smooth muscle cells are first lysed from the surface of the bioreactor actuation membrane using M-PER® Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) to break the cell membrane and solubilise the cytoskeletal protein within the cell membrane. The cell lysate is then collected and centrifuged. The supernatant, which is now saturated with proteins, is then transferred to a new tube collected and frozen at -86°C for further processing.

Upon completion of the experiment, the cell lysate samples would then be tested for protein concentration by using the method developed by Bradford [134]. The cell lysate is mixed with Quick Start Bradford 1× Dye Reagent (Bio-Rad Laboratories, Hercules, CA) and dispensed into a 96 well microplate and measured for their light absorbance at 595nm using a GENios microplate reader. In addition, a standard curve is plotted using Bovine Serum Albumin (BSA) Protein Standard (Sigma, St. Louis, Missouri) and used to quantify the amount of protein present in the cell lysate samples.

To prepare the cell lysate for protein separation, the cell lysate is first diluted 1:1 with 90% Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) and 10% β -Mercaptoethanol. The mixture is heated at 95°C for 4 minutes and allowed to cool to room temperature.

Using the information obtained from the protein quantification, equal amount of total protein from each cell lysate sample is dispensed and separated by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described in [135] using a Mini-PROTEAN® 3 Cell (Bio-Rad Laboratories, Hercules, CA). SDS-PAGE is performed at a constant voltage of 100 Volts for about 70 minutes.

The separated proteins are then transferred from the polyacrylamide gel onto Hybond ECL nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA) at 25 Volts for 12 hours.

After proteins are transferred to the membrane, the membrane is first washed 3 times for 15 minutes each in a washing made up of 1× Phosphate Buffered Saline, pH 7.4 with 0.1% Tween-20 (Sigma, St. Louis, Missouri). Subsequently, the membrane is blocked with 1% Bovine Serum Albumin in washing buffer (blocking buffer) for one hour. After blocking, the primary antibody is added directly to the blocking buffer at a suitable dilution to react and bind to the cytoskeletal protein under investigation for one hour.

The remaining primary antibody is then removed by washing the membrane 3 times for 15 minutes each with the washing buffer. After washing, secondary antibody is added at the appropriate dilution to the washing buffer to bind and react with the primary antibody on the membrane for one hour. After the secondary is incubated for one hour, the membrane is again washed 3 times for 15 minutes each with the washing buffer. ECL Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK) is then added onto the membrane for one minute and removed.

Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK) is then exposed to the membrane to detect the chemiluminescent signal. The exposed Hyperfilm ECL is then developed with Kodak GBX Developer (Eastman Kodak Company, NJ) and fixed with Kodak GBX Fixer (Eastman Kodak Company, NJ).

The developed film is then scanned with a Model GS-800™ Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA) and analysed with Quantity One

1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA) to obtain the relative optical density.

A detailed protocol used for the protein separation via SDS-PAGE and immunoblotting of the separated proteins can be found in Appendix I together with the formulations of the solutions and reagents used in this procedure.

4 RESULTS AND DISCUSSION

4.1 Evaluation of Two-Dimensional Strain Bioreactor System

4.1.1 Thermo Gravimetric Analysis of Bioreactor Actuation Membrane

After the solvent casted polyurethane membrane have been post processed by vacuuming the membrane at room temperature for 7 days, a sample of the membrane is cut and weighed before being analysed by a Thermo Gravimetric Analyser.

The TGA analysis of the fabricated polyurethane membrane is performed at a heating rate of 10°C per minute from room temperature to 500°C to determine percentage of mass loss at a specific temperature. Figure 28 shows the results obtained from the Thermo Gravimetric Analysis of the polyurethane membrane.

Figure 28. Thermo Gravimetric Analysis of solvent casted Chronoflex® AL-80A in 1,4-Dioxane

From the results obtained from the TGA analysis, a weight loss of less than 0.4% by weight can be observed at a heating temperature of 101.6°C, which is the boiling point of the 1,4-Dioxane used as the solvent.

In addition, the weight derivative results shows that there is only an increase in the rate of weight loss of the sample at about 270°C, which is well pass solvent's boiling point of the Chronoflex® AL-80A's glass transition temperature of 210°C.

As such, it can be concluded that the post processing processes of the solvent casted polyurethane membrane would be able to reduce the residual solvent to levels of non-cytotoxicity.

4.1.2 Theoretical Analysis of Bioreactor Actuation Membrane

4.1.2.1 Classical Bending Theory

Based on the classical bending theory, it is assumed that the bioreactor membrane can be modelled as a circular plate with edges clamped. As the membrane is actuated using fluid displacement, it is thus assumed that the membrane is subjected to uniform pressure loading.

For the purpose of the theoretical analysis of the bioreactor membrane, the limitation of the classical bending theory is that it is only valid for small deflections up to half the thickness of the bioreactor membrane [136] and is not suitable for gross deflections exhibited by the hyper-elastic properties of the bioreactor membrane. Additionally, material properties of the bioreactor membrane such as the Young's Modulus and Poisson's ratio must be predetermined.

However, if the bioreactor membrane is modelled as a circular plate with freely supported edges, this would allow for an initial angle of deflection, ϕ , to describe the breakdown of boundary condition at the clamped edges as exhibited by the hyper-elastic bioreactor membrane. Figure 29 shows the bioreactor membrane in its cross-sectional and planar view as used for the theoretical analysis by the classical bending theory.

Figure 29. Cross-sectional and planar representation of bioreactor membrane for theoretical analysis by the classical bending theory

To attempt to model the hyper-elastic bioreactor membrane, the following assumptions must be made:

- Membrane properties and thickness, t , are uniform throughout
- Membrane material is isotropic
- Pressure loading on the membrane is symmetrical about the central axis
- Bending moment in the membrane is always zero

By calculating the equilibrium conditions over a radius r .

For vertical force equilibrium: $\sum F_y = 0$

$$\pi r^2 P = \sigma_r 2\pi r t \sin \phi$$

$$\frac{Pr}{t} = 2\sigma_r \sin \phi \quad (1)$$

For a small element $\delta\theta \delta r$ at $r = r$,

Vertical force equilibrium: $\sum F_y = 0$

$$\begin{aligned}\sigma_r r t \delta\theta \sin\phi + Pr \delta\theta \delta r &= t(\sigma_r + \delta\sigma_r)(r + \delta r) \sin(\phi + \delta\phi) \delta\theta \\ (\sigma_r + \delta\sigma_r)(r + \delta r) \sin(\phi + \delta\phi) &= (\sigma_r r + \delta\sigma_r r + \sigma_r \delta r + \delta\sigma_r \delta r)(\sin\phi + \cos\phi \delta\phi) \\ \frac{Pr}{t} \delta r &= (\sigma_r \delta r + \delta\sigma_r r + \delta\sigma_r \delta r) \sin\phi + (\sigma_r r + \delta\sigma_r r + \sigma_r \delta r + \delta\sigma_r \delta r) \cos\phi \delta\phi\end{aligned}$$

Dividing by δr ,

$$\frac{Pr}{t} = \left(\sigma_r + \frac{\delta\sigma_r}{\delta r} r + \delta\sigma_r \right) \sin\phi + (\sigma_r r + \delta\sigma_r r + \sigma_r \delta r + \delta\sigma_r \delta r) \cos\phi \frac{\delta\phi}{\delta r} \quad (2)$$

Substituting Equation (1) into Equation (2) and ignoring small terms as $\delta r \rightarrow 0$,

$$\sigma_r \sin\phi = \left(\frac{\delta\sigma_r}{\delta r} r \right) \sin\phi + \sigma_r r \cos\phi \frac{\delta\phi}{\delta r} \quad (3)$$

Horizontal force equilibrium: $\sum F_x = 0$

$$t(r + \delta r)(\sigma_r + \delta\sigma_r) \cos(\phi + \delta\phi) \delta\theta + Pr \delta\theta \delta r \tan\phi = r t \sigma_r \cos\phi \delta\theta + \sigma_\theta t \delta r \delta\theta$$

Dividing by t and $\delta\theta$,

$$(r + \delta r)(\sigma_r + \delta\sigma_r) \cos(\phi + \delta\phi) + \frac{Pr}{t} \delta r \tan\phi = r \sigma_r \cos\phi + \sigma_\theta \delta r \quad (4)$$

From trigonometric identities [137],

$$\cos(A + B) = \cos A \cos B - \sin A \sin B$$

Hence,

$$\cos(\phi + \delta\phi) = \cos\phi \cos\delta\phi - \sin\phi \sin\delta\phi = \cos\phi - \sin\phi \delta\phi \quad (5)$$

Substituting Equation (5) into Equation (4) and ignoring small terms as $\delta r \rightarrow 0$,

$$\left(r \frac{\delta\sigma_r}{\delta r} + \sigma_r \right) \cos\phi - (r \sigma_r) \sin\phi \frac{\delta\phi}{\delta r} + \frac{Pr}{t} \tan\phi = \sigma_\theta \quad (6)$$

Moment Equilibrium: $\sum M = 0$

Summing moments from $r \rightarrow (r + \delta r)$,

$$\frac{Pr^2 \delta r \delta \theta}{2} + \frac{P(\delta r \tan \phi)^2 r \delta \theta}{2} = \delta r \tan \phi (tr \delta \theta \sigma_r \cos \phi)$$

Dividing by $\delta \theta$ and δr ,

$$\frac{Pr}{2t} (r + \delta r (\tan \phi)^2) = r \tan \phi \cos \phi \sigma_r = r \sigma_r \sin \phi$$

Ignoring small terms as $\delta r \rightarrow 0$,

$$\frac{Pr^2}{2t} = r \sigma_r \sin \phi$$

$$\frac{Pr}{t} = 2 \sigma_r \sin \phi \quad (1)$$

This final equation is equal to Equation (1) found in page 69 due to the assumptions made in developing this theory. Therefore, due to the non-linear behaviour of the hyper-elastic membranes such as natural rubber and polymeric elastomers [138, 139], a trial shape function would be required to predict the strain and deformation of the membrane as shown in Section 4.1.3..

Thus, the classical bending theory is not suitable to be used to provide an analytical solution to describe the behaviour of the bioreactor membrane under varying deflections.

4.1.2.2 Principle of Virtual Work/Displacement

Using the principle of virtual work/displacement on the bioreactor membrane, it is assumed that the deflection caused by the difference in pressure across both sides of the bioreactor membrane is infinitesimally small. Also, the information about the profile of the deflection as a function of the radius has to be predetermined [140, 141].

However for our purpose of characterising the bioreactor membrane, the deflection of the membrane is not previously known and the deflection caused by the difference across both sides of the membrane is not infinitesimally small. Thus, it is not possible to use the principle of virtual work/displacement to obtain an analytical solution to describe the behaviour of the bioreactor membrane under varying deflections.

4.1.2.3 Geometrical Modelling of Spherical Section

In order to obtain an analytical solution to describe the state of strain within the bioreactor membrane, the bioreactor membrane is geometrically modelled as a spherical section (also known as a spherical cap).

As the spherical section is symmetrical about the central axis, a two dimensional representation of the spherical section is used to obtain an analytical solution to characterise the membrane as shown in Figure 30.

Figure 30. Two dimensional representation of the spherical section (A)

From Figure 30, it can be observed that

$$y_{\max} = \rho - \sqrt{\rho^2 - R^2}$$

$$\rho - y_{\max} = \sqrt{\rho^2 - R^2}$$

Squaring both sides,

$$(\rho - y_{\max})^2 = \rho^2 - R^2$$

Expanding the equation,

$$\rho^2 - 2\rho y_{\max} + y_{\max}^2 = \rho^2 - R^2$$

$$2\rho y_{\max} = y_{\max}^2 + R^2$$

$$\rho = \frac{y_{\max}^2 + R^2}{2y_{\max}} \quad (7)$$

Using coordinate shifting from the centre of radius of curvature to the membrane position,

$$y - \sqrt{\rho^2 - R^2} = \sqrt{\rho^2 - r^2}$$

$$y(r) = \sqrt{\rho^2 - R^2} + \sqrt{\rho^2 - r^2} \quad (8)$$

By substituting Equation (7) into Equation (8),

$$y(r) = \sqrt{\left(\frac{y_{\max}^2 + R^2}{2y_{\max}}\right)^2 - R^2} + \sqrt{\left(\frac{y_{\max}^2 + R^2}{2y_{\max}}\right)^2 - r^2}$$

From factorisation, $a^2 - b^2 = (a + b)(a - b)$,

$$y(r) = \sqrt{\left(\frac{y_{\max}^2 + R^2}{2y_{\max}} + R\right)\left(\frac{y_{\max}^2 + R^2}{2y_{\max}} - R\right)} + \sqrt{\left(\frac{y_{\max}^2 + R^2}{2y_{\max}}\right)^2 - r^2}$$

$$y(r) = \sqrt{\left(\frac{y_{\max}^2 + R^2 + 2y_{\max}R}{2y_{\max}}\right)\left(\frac{y_{\max}^2 + R^2 - 2y_{\max}R}{2y_{\max}}\right)} + \sqrt{\left(\frac{y_{\max}^2 + R^2}{2y_{\max}}\right)^2 - r^2}$$

From factorisation, $(a + b)^2 = a^2 + 2ab + b^2$ and $(a - b)^2 = a^2 - 2ab + b^2$,

$$y(r) = \sqrt{\frac{(y_{\max} + R)^2 (y_{\max} - R)^2}{4y_{\max}^2}} + \sqrt{\left(\frac{y_{\max}^2 + R^2}{2y_{\max}}\right)^2 - r^2}$$

$$y(r) = \sqrt{\left(\frac{(y_{\max} + R)(y_{\max} - R)}{2y_{\max}}\right)^2} + \sqrt{\left(\frac{y_{\max}^2 + R^2}{2y_{\max}}\right)^2} - r^2$$

$$y(r) = \frac{(y_{\max} + R)(y_{\max} - R)}{2y_{\max}} + \sqrt{\left(\frac{y_{\max}^2 + R^2}{2y_{\max}}\right)^2} - r^2$$

Again, from factorisation, $a^2 - b^2 = (a + b)(a - b)$,

$$y(r) = \frac{y_{\max}^2 - R^2}{2y_{\max}} + \sqrt{\left(\frac{y_{\max}^2 + R^2}{2y_{\max}}\right)^2} - r^2 \quad (9)$$

Engineering strain is defined as the ratio of elongation or change in length (ΔL) to original length (L)

$$e = \frac{\text{elongation}}{L} = \frac{\Delta L}{L} \quad (10)$$

Thus, in order to determine the strain in the radial direction, e_r , we have

$$e_r = \frac{\text{elongation}}{R} \quad (11)$$

From Figure 30, it can be observed that the deflected radial length can be represented by $\rho\alpha$, thus,

$$\text{Elongation} = \rho\alpha - R \quad (12)$$

By substituting Equation (12) into Equation (11),

$$e_r = \frac{\rho\alpha - R}{R} = \frac{\rho\alpha}{R} - 1 \quad (13)$$

Also, from Figure 30, it can be observed that

$$\sin \alpha = \frac{R}{\rho}$$

$$\alpha = \sin^{-1}\left(\frac{R}{\rho}\right) \quad (14)$$

Again, substituting Equation (7) into Equation (14),

$$\alpha = \sin^{-1} \left(\frac{2y_{\max}R}{R^2 + y_{\max}^2} \right) \quad (15)$$

Thus, by substituting Equation (15) into Equation (13),

$$e_r = \frac{\frac{y_{\max}^2 + R^2}{2y_{\max}} \sin^{-1} \left(\frac{2y_{\max}R}{R^2 + y_{\max}^2} \right)}{R} - 1$$

$$e_r = \frac{y_{\max}^2 + R^2}{2y_{\max}R} \sin^{-1} \left(\frac{2y_{\max}R}{R^2 + y_{\max}^2} \right) - 1 \quad (16)$$

From Figure 31, it can be observed that upon deflection of the membrane, the arbitrary point, r , on the undeflected membrane would be displaced radially outwards from the central axis to a new position r' .

Figure 31. Two dimensional representation of the spherical section (B)

Based on the definition of engineering strain from Equation (10), the tangential strain, e_θ , caused by the deflection, can be described as the ratio of change in circumference to the original circumference. is as shown below.

$$e_\theta(r) = \frac{2\pi r' - 2\pi r}{2\pi r} = \frac{r' - r}{r} \quad (17)$$

From Figure 31, it can be observed that

$$r' - r = AB \sin \beta \quad (18)$$

Substituting Equation (18) into Equation (17), we get,

$$e_{\theta}(r) = \frac{AB \sin \beta}{r} \quad (19)$$

Also, from Figure 31, it can also be observed that

$$OB = \sqrt{\rho^2 - R^2 + r^2} \quad (20)$$

and,

$$\sin \beta = \frac{r}{OB} \quad (21)$$

and,

$$AB = \rho - OB \quad (22)$$

Substituting Equation (21) and Equation (22) into Equation (19), we get

$$\begin{aligned} e_{\theta}(r) &= \frac{\rho - OB}{r} \left(\frac{r}{OB} \right) \\ e_{\theta}(r) &= \frac{\rho}{OB} - 1 \end{aligned} \quad (23)$$

Substituting Equation (7) and Equation (20) into Equation (23), we get

$$\begin{aligned} e_{\theta}(r) &= \frac{\rho}{\sqrt{\rho^2 - R^2 + r^2}} - 1 \\ e_{\theta}(r) &= \frac{\frac{y_{\max}^2 + R^2}{2y_{\max}}}{\sqrt{\left(\frac{y_{\max}^2 + R^2}{2y_{\max}} \right)^2 - R^2 + r^2}} - 1 \\ e_{\theta}(r) &= \frac{\frac{y_{\max}^2 + R^2}{2y_{\max}}}{\sqrt{\frac{y_{\max}^4 + 2y_{\max}^2 R^2 + R^4 - 4y_{\max}^2 R^2 + 4y_{\max}^2 r^2}{(2y_{\max})^2}}} - 1 \end{aligned}$$

$$e_{\theta}(r) = \frac{y_{\max}^2 + R^2}{\sqrt{y_{\max}^4 + 2y_{\max}^2 R^2 + R^4 - 4y_{\max}^2 R^2 + 4y_{\max}^2 r^2}} - 1$$

$$e_{\theta}(r) = \left(\frac{y_{\max}^2 + R^2}{\sqrt{R^2 - y_{\max}^2 + 4y_{\max}^2 r^2}} \right) - 1 \quad (24)$$

To find the change in volume (ΔV) caused by the any given maximum deflection ($y_{\max}$), the volume of the spherical section is given by [142],

$$\Delta V = \frac{1}{6} \pi y_{\max} (3R^2 + y_{\max}^2) \quad (25)$$

$$\Delta V = \pi y_{\max} \left(\frac{R^2}{2} + \frac{y_{\max}^2}{6} \right) \quad (26)$$

Thus, from the modelling of a spherical section, we are able to obtain an analytical solution to characterise the behaviour of the membrane under varying deflections.

Equations (16) and (24) are plotted in Figure 32 for a nominal displacement $y_{\max}$ of $0.1 \times R$. The radial strain for the bioreactor is constant while the tangential strain displays a parabolic profile, with $e_{\theta} = -0.5$ at $r = R$.

Figure 32. Profile for strain in bioreactor for $y_{\max} = 0.1R$ by geometrical modelling of a spherical section

Similar results of radial and tangential strain profile were previously obtained by Williams *et al.* [102] using the method of large plate deflection theory, with the exception of the simplification of the analytical solution using a spherical section modelling which neglects the boundary effects. The solution presented in Figure 32 cannot be completely correct as all the radial strains on the membrane would have to pass through the central axis of the bioreactor well, thereby acting as the tangential strain for the centre point within the bioreactor. As such, the values of both the radial strain and the tangential strain would have to be equal to be correct.

However, in another solution presented in the same paper using the method of large deformations of thin elastomeric membranes, a trivial solution was obtained through the use of an assumed shape function.

The results presented using the trivial solution showed that with the radial strain and tangential strain are equal in magnitude at the centre of the bioreactor well. When edge effects are neglected, the radial strain is constant throughout along the radius of the bioreactor well, while the tangential strain decreases parabolically from the maximum value of the constant radial strain to zero at the well edge. Figure 33 shows the strain profile as presented by Williams *et al.* [102].

Figure 33. Profile for strain in bioreactor as presented by Williams *et al.* [102]

The radial strain calculated from the use of the assumed shape function was also found to be in good agreement with the radial strain obtained by the ratio of difference in chord and arc length to the chord length [143, 144], which is conceptually identical to the radial strain equation that is presented by the geometrically modelling of the spherical section found in this report. As such, the strain as predicted by Equation (16) using geometrical modelling of the spherical section would be correct. This solution was further verified experimentally and found to be a good match with the proposed trivial solution.

Due to the complex behaviours of the thin hyperelastic membrane when undergoing large deformation in such a configuration, the simplified geometrical model presented in this thesis was not sufficient to correctly predict the tangential state of strain on the bioreactor membrane. However, the general trend of the tangential strain being at a maximum and equal to the radial strain magnitude at the well centre and parabolically decrease along the radius are correct. Further research would be required to correctly predict the tangential strain using the spherical section model.

Since the radial strain within the bioreactor membrane is constant and is also indicative of the maximum tangential strain, at the centre of the bioreactor well, the strain used to quantify the amount of mechanical stimulation the oesophageal smooth muscle cells receive from the bioreactor would be the radial strain as predicted by Equation (16).

As the bioreactor's displacement is designed to be hydraulically actuated, hence the relationship between maximum displacement ($y_{\max}$) and the increase in volume of the chamber, is given by Equation (26) and is used as a basis for the calculation of mechanical strain.

Figure 34. Variation of change in volume with varying deflection

The variation of increase in volume corresponding to the maximum deflection can be obtained from Equation (26). Figure 34 shows the relationship between the maximum deflection $y_{\max}$ and the increase in volume, up to the theoretical limit for this particular bioreactor design where Equation (26) no longer holds true, which occurs when the maximum deflection exceeds the well radius. As can be seen from Figure 34, the relationship is close to linear in the range of deflections used in this experiment due to the dominance of the $R^2 y_{\max}$ term in Equation (26) for the range of experimental deflections.

4.1.3 Results of Experimental Investigation of Bioreactor Actuation Membrane

The coordinates obtained experimentally by the Coordinate Measuring Machine (CMM) were used to plot the deflected profile of the bioreactor membrane for a range of maximum deflections. The different maximum deflections were obtained by injecting amounts of actuation fluid into the bioreactor test cell to provide different volumetric displacements. Using the experimental data obtained from the CMM line scans, the profile of the membrane deflection can be obtained by plotting the deflection against the radius of the bioreactor well.

Figure 35 to Figure 39 show the experimentally obtained deflection profile plotted with the theoretical shape profile as predicted by Equation (9) from the geometrical modelling of a spherical section as presented in Section 4.1.2.3.

Figure 35. Profile of membrane deflection in bioreactor for $y_{\max}=0.645\text{mm}$

Figure 36. Profile of membrane deflection in bioreactor for $y_{\max}=1.226\text{mm}$

Figure 37. Profile of membrane deflection in bioreactor for $y_{\max}=2.262\text{mm}$

Figure 38. Profile of membrane deflection in bioreactor for $y_{\max}=3.308\text{mm}$

Figure 39. Profile of membrane deflection in bioreactor for $y_{\max}=4.229\text{mm}$

From Figure 35 to Figure 39, it can be observed that the deflected profile of the bioreactor membrane obtained experimentally was very similar to the profile of a spherical cap/section. However for Figure 35 and Figure 36, the amount of variation (percentage error) between the measured deflection and the theoretical value is large. This may be attributed to the small value of the deflection in these experiments, where any errors caused by the measurement technique will be proportionally large. Errors in the measured values can be ascribed to:

1. At small deflections, vibrations were found to have a significant effect on the accuracy of readings.
2. The measurement technique relies upon a laser reflecting off the surface of the membrane. In these tests, the vinyl membrane used is translucent and for measurements where the angle of incidence of the laser is high, internal reflection may cause erroneous measurements.
3. There is some variation in the thickness and surface roughness of the membrane material used in these experiments.

Figure 40. Profile of membrane deflection in bioreactor for varying $y_{\max}$

Figure 40 shows the local deflections along the well radius when subjected to various maximum deflections ($y_{\max}$) at the well centre.

From the experimental findings obtained for the two-dimensional strain bioreactor, it is observed that the deflection profile of the bioreactor membrane coincides with the deflection shape as predicted by the geometrical modelling of the spherical section as presented in Section 4.1.2.3. As such, the two-dimensional mechanical strain bioreactor design has been well characterised and understood.

Using the same working principles as the first prototype of the two-dimensional mechanical strain bioreactor developed in the first year of study, a six-well variant of the bioreactor have been fabricated would be used in the subsequent cell experiments. The detailed design for the six-well two-dimensional mechanical strain bioreactor can be found in Appendix C.

4.2 Results of Characterisation of Primary Oesophageal Smooth Muscle Cells

Using the procedures described in Section 3.2.3 and Appendix G, immunocytochemical staining is carried out on the isolated porcine oesophageal smooth muscle cells one day after their isolation. A negative control is prepared for each different primary antibody used for immunocytochemical staining to determine the degree of non-specific staining present in each antibody system.

Figure 41. Immunocytochemical staining of cytoskeletal protein A. smooth muscle α -actin B. desmin C. smooth muscle myosin heavy chain (40 $\times$ objective)

Figure 41 shows the immunocytochemical staining results of the cytoskeletal protein. On the left side, the cytoskeletal proteins were probed for by the primary antibodies and on the right, the primary antibodies were replaced with negative control IgG to determine the amount of non specific staining between the secondary antibodies and the oesophageal smooth muscle cells.

From the immunocytochemical staining results obtained from the primary antibodies, it can be seen that the stains form thin sharp filamentous lines which is characteristic of cytoskeletal proteins when observed by indirect immunofluorescence.

However, when compared to the immunocytochemical staining results obtained for the negative control IgG in place of the primary antibodies, the stains observed are diffuse, and low in intensity, indicating random and non-specific binding of secondary and tertiary antibodies to cellular proteins.

As such, it can be concluded that the freshly isolated oesophageal cell are indeed smooth muscle cells based on the results of the immunocytochemical staining.

4.3 Results of Cytocompatibility of Bioreactor Actuation Membrane

4.3.1 Cell Proliferation Test Results

Using the method as described in Section 3.3.1, the proliferation of porcine oesophageal smooth muscle cells on oxygen plasma treated polyurethane membrane were investigated.

Figure 42 shows the results of the cell proliferation test of oesophageal smooth muscle cells on the oxygen plasma treated polyurethane membrane.

Figure 42. Proliferation of Oesophageal Smooth Muscle Cells on Oxygen Plasma Treated Polyurethane membrane over 14 days

From the results as shown in Figure 42, porcine oesophageal smooth muscle cells are able to attach onto the oxygen plasma treated polyurethane membrane and proliferate. After 14 days, the smooth muscle cells remain attached on the polyurethane membrane and have grown to form a confluent monolayer covering the entire membrane.

Figure 43 shows porcine oesophageal smooth muscle cells forming a confluent monolayer on the oxygen plasma treated polyurethane membrane on day 14.

Figure 43. Confluent monolayer of porcine oesophageal smooth muscle cells growing on oxygen plasma treated polyurethane membrane on day 14 (10× objective)

Thus, from the cell proliferation experiment, it can be concluded that the oxygen plasma treated polyurethane membrane is non-cytotoxic and is a suitable substrate for use as the bioreactor actuation membrane. In addition, after oxygen plasma surface treatment, it can also serve as a two dimensional scaffold for use in planar mechanobiology work.

4.3.2 Cell Attachment Test Results

Using the same methods and parameters described in Section 3.3.1 for the cell proliferation test, 50000 oesophageal smooth muscle cells or 5200 cells/cm² were seeded onto the different substrates. The cells were then incubated overnight to allow attachment.

Figure 44 shows the results of the cell attachment test of the 4 different substrates used for the test.

Figure 44. Attachment of Oesophageal Smooth Muscle Cells on different substrates

From the results of the cell attachment test as shown in Figure 44, it can be seen that there is no significant difference between the cell attachment efficiency of the oesophageal smooth muscle cells onto the oxygen plasma treated polyurethane membrane as compared to the other common extracellular matrix proteins used in cell culture to enhance the attachment of the cells after overnight incubation.

Thus, it can be concluded that the oxygen plasma treated polyurethane membrane surface is able to provide a similar quality of attachment for the cells compared to other more commonly used cell attachment surfaces.

4.4 Effects of Cell Response to Two-Dimensional Mechanical Strain Stimulation

Two-dimensional mechanical stimulation was applied to the oesophageal smooth muscle cells-oxygen plasma treated polyurethane membrane as described in Section 3.4 to study the effects of mechanical stimulation on cell-scaffold constructs.

The oesophageal smooth muscle cells were serially sub-cultivated using the procedures as described in Section 3.2.2 and Appendix G on tissue culture polystyrene (TCPS) to passages 4 to 6 before being used for the mechanical stimulation experiments. Oesophageal smooth muscle cells were then dissociated from TCPS and seeded onto the polyurethane membrane at 2.5×10^5 cells per well ($\sim 2.6 \times 10^4$ cells/cm²). The cells are then left to attach for 2 days before the commencement of mechanical stimulation.

For the purpose of this project, 0% (static control), 2% and 5% radial strains are investigated at a frequency of 5 cycles per minute and 420 cycles a day. The mechanically stimulated cells are then assessed for the cellular response using the following methods as to be discussed below.

4.4.1 Cell Alignment

When subject to two-dimensional mechanical strain using the bioreactor designed to facilitate this study, oesophageal smooth muscle cells are subjected to a complex state of strain imparted from the bioreactor actuation membrane.

As discussed previously in Section 4.1.2.3, the state of strain on the bioreactor actuation membrane would be a combination of two principal strains, namely the radial strain and the tangential strain. At the centre of the bioreactor well, the strain experienced by the cells would be equi-biaxial, where the radial strain and the tangential strain would be equal. The radial strain on the membrane would be constant throughout from the central axis of the bioreactor well to the edge. However, the tangential strain would decrease parabolically from the centre of the well to the edge to zero, where there is only the presence of the radial strain.

Based on the previous work on one-dimensional mechanical strain, smooth muscle cells are known to align perpendicularly to the direction of strain [28, 31-33, 117, 126, 145]. However, no previous works have investigated the effects of smooth muscle cell alignment for two-dimensional mechanical strain under different strain ratios.

The phase contrast micrographs of 3 areas of the bioreactor well, namely the well centre, mid radius and well edge are presented for 0% (control), 2% and 5% radial strain. The cell-scaffold constructs were harvested from the bioreactors after 3 days of mechanical stimulation using the cyclic strain regime as described in Section 3.4.

Figure 45 shows the state of strain present on the polyurethane membrane as experienced by the oesophageal smooth muscle cells at the 3 locations of interest in the bioreactor well.

Figure 45. Profile of state of strain at the 3 location of interest within the bioreactor

Figure 46 presents the results of the 0% (control) and 2% radial strain samples after two-dimensional strain mechanical stimulation for the after 3 days.

Figure 46. Phase Contrast Micrographs of oesophageal smooth muscle cells at different well locations under different mechanical strain (20× objective)

From the phase contrast micrographs presented in Figure 46, it can be observed that the control experiment exhibit random alignment and the normal hill and valley growth pattern when it reaches confluence in static culture in all 3 locations of the bioreactor well. Although domains of local alignment of the smooth muscle cells can be observed, the cell alignment is still random on a macro scale. This phenomenon is also exhibited by smooth muscle cells when cultured on normal tissue culture treated surfaces [146, 147].

Oesophageal smooth muscle cells that have been stimulated with 2% radial strain in the two-dimensional strain bioreactor exhibits different orientation at the 3 different locations.

At the edge of the bioreactor well, the smooth muscle cells are observed to align themselves perpendicular to the radial strain in the absence of the tangential strain. This observation corresponds to previous reported works on smooth muscle cells being subjected to uniaxial cyclic mechanical strain stimulation, where smooth muscle cells are known to align themselves perpendicular to the direction of mechanical strain [28, 31-33, 117, 126, 145].

Buck [145] postulated that the cellular response of smooth muscle cells aligning perpendicular to the direction of strain when uniaxially stimulated is to minimise mechanical stresses. The perpendicular alignment of the smooth muscle cell would move the long axis of the cell from the direction of strain so as to preserve their existing focal adhesion points.

At the mid-radius of the well, the cells are observed to still predominantly align itself perpendicularly to the constant radial strain. However, cells that are aligned parallel to the direction of radial strain can now be observed. The presence of cells aligning parallel to the radial strain is possibly due to the presence of the tangential strain component which is increasing from the edge of the bioreactor to the well centre.

In such a situation where both radial strain and tangential strain are present, the smooth muscle, in order to minimise mechanical stresses on itself, would have to align itself perpendicularly to one of the principal strains. As the strain state at the mid-radius of the bioreactor well has a larger radial strain than its tangential strain, more smooth muscle cells are observed to still align themselves perpendicular to the radial strain than the tangential strain for more efficient minimisation of mechanical stress experienced.

At the centre of the bioreactor well, smooth muscle cells are observed to align themselves perpendicular to each other in equal numbers. At this location, both the

radial strain and tangential strain are equal, thereby creating an equi-biaxial state of strain.

From the geometrical perspective, the equi-biaxial state of strain is created by all the radial strains passing through the central axis of the bioreactor well. As such, at the fringes of the area surrounding the central axis of the bioreactor well, oesophageal smooth muscle cells can be observed to align themselves parallel to the radial strain radiating outwards from the central axis as the state of strain just outside the region surrounding the central axis would have a smaller tangential strain as compared to the radial strain.

The cell alignment results for the oesophageal smooth muscle cells after 3 days of two-dimensional mechanical stimulation at 5% radial strain is presented in Figure 47 along with a phase contrast micrograph of the polyurethane membrane. (Please note that the direction towards the bioreactor well centre is at the top left hand corner of the phase contrast micrographs.)

Figure 47. Phase Contrast Micrographs of oesophageal smooth muscle cells at A. Centre B. Mid-Radius C. Edge D. PU Membrane after 5% radial strain for 3 days (40× objective)

From the results of the 5% radial strain presented in Figure 47, few cells were found to have remained attached to the polyurethane membrane. This is probably due to the poor attachment that cells have on smooth substrates such as polyurethane and silicone rubber when subjected to large uniaxial strain magnitudes or high frequencies of cyclic strain [145]. However, for the case of cyclic biaxial strain, no studies have been previously reported. As such, it is unknown if smooth muscle cells would be able to withstand a higher or lower strain magnitude in the case of biaxial strain.

The cellular alignment of the oesophageal smooth muscle cells that were subjected to 5% radial strain showed similar results when compared to cells subjected to 2% radial strain. At the well centre, oesophageal smooth muscle cells were found to be connected to each other at approximately right angles. Some cells were found to exhibit the spreading morphology known to be exhibited by synthetic phenotype [146] although most cells are spindle shaped indicating a hypercontractile phenotype [148] and has a highly condensed cytoplasm as compared to the oesophageal smooth muscle cells after 2% radial strain stimulation.

At the mid-radius of the bioreactor well, cells were found to align both parallel and perpendicular to the radial strain just as observed in the 2% strain experiment. More cells were found to align parallel to the radial strain nearer the centre of the well. The morphology of the smooth muscle cells are mostly spindle like and highly bi-polar. The cells observed in this region also connect themselves at about right angles.

At the edge of the bioreactor well, most cells are aligned perpendicular to the radial strain. The morphology of the cells exhibited is also of the contractile phenotype.

Normally, in a situation where the cells are sub confluence under standard cell culture conditions, it is unlikely that the smooth muscle cells will exhibit the contractile phenotype unless cultured on specific extracellular matrix proteins which are known to induce differentiation, or the usage of special culture medium. As such, the results observed from these experiments do suggest that mechanical strain does indeed induce differentiation of smooth muscle cells.

Figure 47D shows the polyurethane membrane when observed under a phase contrast microscope. The formation of the patterns on the polyurethane membrane is caused by the crystallisation of the polyurethane when the hard and soft segments of the polymer phase separates on a micro scale, thereby forming domains [149]. These domains, when observed under a phase contrast microscope, would cause the crystal structures to be revealed due to the different refractive index between the hard and soft segments of the polymer blend causing a phase shift of the light collected at the eyepiece.

4.4.2 Cell Proliferation

To investigate the effects that the cyclic two-dimensional strain mechanical stimulation has on oesophageal smooth muscle cells, a cell proliferation assay was conducted using the CellTiter96® AQueous One Solution Reagent using the procedures described in Section 3.5.2. Due to the massive amount of cell loss from the 5% radial strain mechanical stimulation samples, it would not be possible to perform a cell proliferation assay using the little remaining cells.

As such, oesophageal smooth muscle cells from both 0% (static control) and 2% radial strain were investigated at time points of Day 0, 1, 2 and 3. Day 0 denotes the state of the smooth muscle cells just before the commencement of the cyclic mechanical strain stimulation, after 2 days of incubation for the cells to attach after seeding onto the oxygen plasma treated polyurethane membrane.

The results of the MTS cell proliferation assay conducted on the oesophageal smooth muscle cells are presented in Figure 48.

Figure 48. MTS Cell proliferation assay results between two-dimensional strain mechanical stimulation and control

From the results presented in Figure 48, the absorbance value of the oesophageal smooth muscle cells subjected to cyclic two-dimensional strain mechanical stimulation is constantly lower than that of the static control.

As CellTiter96® AQueous One Solution Reagent is designed to assess the cell number of cultured cells through the metabolic reduction of the MTS tetrazolium compound into a coloured formazan product, any type of changes in the metabolism of the cultured cells would affect the amount of formazan coloured product. The metabolism of the culture cells can change in 2 ways, namely, a change in the number of cells present, or a change in metabolism of the cells through phenotype modulation (i.e. from synthetic phenotype to contractile phenotype).

From the observation of the oesophageal smooth muscle cells after two-dimensional strain mechanical stimulation at 2% radial strain, cells are found to be detached predominantly from the polyurethane membrane at the mid-radius location of the bioreactor well as shown in Figure 49A.

Figure 49. Loss of cells at mid-radius of bioreactor well A. 10× objective B. 20× objective

It was found that certain regions within the bioreactor well (Figure 49B) contained apoptotic cells (indicated by black arrows) that are highly refractive at the cell edges with highly condensed nucleus and cytoplasm. Two-dimensional strain mechanical stimulation studies conducted using both two-dimensional [108] and three dimensional [150] cell-scaffold constructs have reported cell death. In addition, oesophageal smooth muscle cells exhibiting synthetic phenotype, through their well spread morphology, are observed at the fringes next to the areas of massive cell loss, presumably trying to proliferate into these vacated areas.

It is possible that some cells may be adversely affected by the differential biaxial strains experienced at the mid-radius area, thereby resulting in massive cell loss either through apoptosis or the loss of focal adhesion contact during cellular realignment as a result of the biaxial state of strain present on the polyurethane membrane.

Elsewhere on the membrane, large areas of oesophageal smooth muscle cells can be observed at the mid radius areas, aligned perpendicular to the radial strain direction as shown in Figure 50A.

Figure 50. Aligned cells at mid-radius of bioreactor well A. 10× objective B. 40× objective

Upon observation of the cells at the mid-radius areas as shown in Figure 50B, the aligned oesophageal smooth muscle cells are found to be spindle-like and highly bipolar. This morphology exhibited by the smooth muscle cells is indicative of the contractile phenotype.

With the presence of different cell phenotypes present in the bioreactor well being assessed together using the MTS tetrazolium compound, the results obtained from this cell proliferation assay would be an indicator of the overall metabolic state of the cultured oesophageal smooth muscle cells.

The results of the MTS cell proliferation assay presented in Figure 48 can be interpreted in the following way. On the onset of two-dimensional mechanical strain stimulation oesophageal smooth muscle cells with weak focal contact adhesions are detached from the polyurethane membrane, resulting in a lower MTS absorbance intensity as compared to the static control.

After one day of mechanical stimulation, the cells neighbouring the areas of cell loss starts to dedifferentiate into the synthetic phenotype to undergo cell division to replace the detached cells. In addition, cells that are firmly attached to the polyurethane membrane starts to orientate their alignment perpendicularly to one of the principle strains present to minimise mechanical stresses experienced by the cells.

After the second day of mechanical stimulation, the oesophageal cells which are firmly attached to the polyurethane membrane would have realigned perpendicular to one of the principle strains and starting to differentiate. Cells which are next to areas of cell lost would most likely to be proliferating, resulting in a rise in the value of MTS absorbance intensity as compared to the static control.

After the third day of mechanical stimulation, most cells would have already been realigned perpendicular to one of the principle strains and more cells would have been differentiated, resulting in a slightly lower MTS absorbance intensity due to the overall more differentiated state of the cells within the bioreactor well.

Although after 3 days of two-dimensional strain mechanical stimulation, large areas of aligned oesophageal smooth muscle cells which are spindle-like and bipolar are observed to be present on the polyurethane membrane, the process of phenotype modulation of cell differentiation can only further investigated by cytoskeletal protein analysis to obtain a more indicative conclusion which is suggested by the MTS cell proliferation assay.

4.4.3 Cytoskeletal Protein Analysis

To determine the effects of two-dimensional strain mechanical have on oesophageal smooth muscle cell differentiation, cytoskeletal protein markers known to be exhibited by differentiated smooth muscle cells are detected with their respective antibodies. Cell lysate samples were obtained from both the static control and mechanically stimulated oesophageal smooth muscle cells using cell lysis buffer.

The extracted cell lysate samples were first assessed using the Bradford protein assay to determine their protein concentrations. A protein concentration plot is obtained using a BSA protein standard and compared against the cell lysate samples to determine their respective protein concentrations.

Figure 51 shows the absorbance intensity readings of the BSA protein standard used to determine the protein concentration of the cell lysate samples.

Figure 51. BSA protein concentration plot

After the protein concentration of each cell lysate sample is determined, the cell lysate samples separated using SDS-PAGE. 10µg of total cell lysate protein is loaded into each well and separated at 100 Volts for about 75 minutes. After the cytoskeletal proteins in the cell lysate samples were separated, the separated proteins in the polyacrylamide gel were then transferred to a nitrocellulose membrane at 25 Volts overnight.

After the transfer of the separate proteins to the nitrocellulose membrane has been completed, the membrane was then stained with primary antibodies. Primary antibodies staining for smooth muscle α -actin (Dako, Glostrup, Denmark), desmin (Chemicon International, Inc., Temecula, CA), calponin 1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), smooth muscle myosin heavy chain (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and vimentin (AbD Serotech, Oxford, UK) was performed using the cell lysate samples. Figure 52 shows the results of the immunoblotting of the cytoskeletal proteins.

Figure 52. Expression of cytoskeletal proteins

From the immunoblotting results presented in Figure 52, it can be seen that cytoskeletal proteins which indicate differentiation, such as smooth muscle α -actin [132, 148, 151], desmin [132, 152, 153] and calponin 1 [131, 150, 154], are down-regulated upon the commencement of two-dimensional strain mechanical stimulation as compared to the static control. However, these differentiation markers are progressively up-regulated on the second and third day of mechanical stimulation. Smooth muscle myosin heavy chain (SM-MHC) [148, 151] is also investigated by immunoblotting. However, no significant up-regulation of its expression can be observed throughout the experiment.

Vimentin is considered to be a dedifferentiation marker to indicate a more fibroblastic phenotype [132, 150]. It can also be considered to be a mesenchymal cell marker as its expression can be found diversely in cells of the mesenchymal origin [155, 156].

However, the expression of vimentin is not exclusive to cells of mesenchymal origin. Epithelial cells are also found to express vimentin as an intermediate filament in addition to their own epidermal keratins [155-158]. The expression of vimentin in cells also indicates motility [155, 157], when they are stimulated to move and contract under conditions of tissue remodelling. The exact functions of vimentin are yet to be clearly defined although it is suggested that they might be required to provide mechanical support for the nucleus.

Although smooth muscle cells found in the digestive tract is considered rich in desmin content comparatively to its vimentin content, smooth muscle cells are known to co-express both desmin and vimentin when cultured *in vitro* [156], possibly due to the differences in environment (two-dimensional versus three-dimensional) and also the differences or lack of extracellular matrix proteins.

From the cytoskeletal protein analysis of vimentin as shown in Figure 52, basal levels of vimentin can be detected by immunoblotting in the cultured oesophageal smooth muscle cells. The expression levels of vimentin after the onset of two-dimensional strain mechanical stimulation are similar to that as observed by the static control. However, on the second and third day of mechanical stimulation, vimentin expression levels are observed to be up-regulated. This is likely to be as a result of the mechanical stimuli provided by the cyclic two-dimensional strain which is responsible for the realignment of smooth muscle cells perpendicular to one of the principle strains present on the polyurethane membrane. In addition, the presence of synthetic smooth muscle cells near the areas of cell loss (Figure 49) would also contribute to the increase of vimentin expression through the process of dedifferentiation and motility.

Thus, from the results obtained from the cytoskeletal protein analysis by immunoblotting, it can be concluded that cyclic two-dimensional strain mechanical stimulation does promote cell/tissue remodelling as well as induce differentiation to a more contractile phenotype.

Investigation of the effects of two-dimensional strain mechanical stimulation on oesophageal smooth muscle cells has revealed that different strain magnitudes can result in different outcomes on cell-scaffold constructs. High magnitudes of strain may result in massive cell loss through apoptosis, and cell detachment, while strain at an appropriate magnitude is able to induce cell/tissue remodelling by the realignment of the smooth muscle cells and change in phenotype.

Oesophageal smooth muscle cells are observed to align both perpendicular and parallel to the radial strain when mechanically stimulated with two-dimensional strain. Increasing proportions of cells were found to be aligned parallel to the radial strain with the increase of tangential strain towards the bioreactor well centre, indicating that

it may be possible to form a biaxially aligned smooth muscle cell sheet resembling the longitudinal and circumferential alignment of the muscle layer of the oesophagus wall *in vivo* using biaxial mechanical stimulation.

The light absorbance intensity of the MTS cell proliferation assay is consistently lower for the mechanically stimulated cells when compared to the static control. This may be attributed to the loss of cells from the cell-scaffold construct within the bioreactor, and contributed to in part by a change in cell metabolism as cells differentiate from the synthetic to the contractile phenotype as a result of stimulation by two-dimensional strain.

Cytoskeletal protein analysis of the mechanically stimulated oesophageal smooth muscle cells revealed abrupt down-regulation of known differentiation markers for smooth muscle cells such as smooth muscle α -actin, desmin and calponin 1 at the onset of mechanical stimulation. These differentiation markers were then progressively up-regulated as stimulation proceeded. Vimentin, which is thought to be a dedifferentiation marker, is also expressed in a similar manner as the differentiation markers investigated. This result suggests that vimentin should also be considered a marker of cell motility during cell/tissue remodelling in addition to its role as a dedifferentiation marker.

5 CONCLUSION

The objective of this project is to study the effects of two-dimensional mechanical strain stimulation on oesophageal cell types for the purpose of tissue engineering a clinically functional bioartificial oesophagus. Porcine oesophageal smooth muscle cells are chosen as the focus of the study due to the previously known responses to one-dimensional strain mechanical stimulation.

A bioreactor system capable of two-dimensional mechanical strain stimulation was designed and fabricated along with a bioreactor actuation membrane that is both non-cytotoxic and is able to function as a two-dimensional scaffold to support cell growth directly on its surface after oxygen plasma treatment.

The characterisation of the state of strain on the bioreactor actuation membrane during bioreactor operation is performed. The profile of the bioreactor actuation membrane is investigated at various stages of deflection using a coordinate measuring machine and the shape function of the deflected bioreactor actuation membrane is derived through the geometrical modelling of a spherical section. Both the radial and tangential strains equations were obtained using the derived shape function. The radial strain on the bioreactor actuation membrane can be correctly predicted by the derived equation. The tangential strain equation derived using the deflection shape function is unable to predict the tangential strain on the bioreactor actuation membrane correctly. However, the derived tangential strain equation does give the trend of the maximum strain magnitude at the centre of the bioreactor well, decreasing parabolically to the minimum at the well edge. Previous work on characterisation of the state of strains on similar systems by Winston *et al.* [143] and Williams *et al.* [102] concludes that the radial strain equation presented in this report accurately predicts the state of radial strain on the bioreactor actuation membrane, whereas the tangential strain equation derived is only able to predict its trend.

For the study of the effects of two-dimensional strain on oesophageal smooth muscle cells, a primary cell line of porcine oesophageal smooth muscle cells was successfully isolated and characterised by immunocytochemical staining. Cell attachment and

proliferation studies were conducted using the isolated smooth muscle cells on oxygen plasma treated polyurethane membrane, which is to be used as a two-dimensional scaffold in this study.

Oesophageal smooth muscle cells were mechanically stimulated with two-dimensional strain for 3 days at 0% (static control) 2% and 5%. The mechanically stimulated cells were then evaluated by their cellular alignment, cell proliferation and cytoskeletal protein expression.

Oesophageal smooth muscle cells were demonstrated to align perpendicular to both principle strains when stimulated biaxially in both 2% and 5% radial strain. At the edge of the bioreactor wells, cells were found to be predominantly aligned perpendicular to the radial strain, where there is near absence of tangential strain. However, at the mid-radius of the well, the cells were both aligned perpendicularly and parallel to the radial strain. This is possibly due to the increase presence of tangential strain at the location. At the centre of the well, where the state of strain is equibiaxial, cells were found to align at about right angles to each other, forming a cell layer resembling the longitudinal and circumferential muscle layer found in the oesophagus wall. It can be concluded that biaxial strain does promote cellular alignment to a more physiological state, and that the cells display a protective response to high magnitudes of strain [145].

Two-dimensional mechanical strain also does not promote proliferation of the smooth muscle cells as indicated from the results of the MTS cell proliferation assay. Observation of the oesophageal smooth muscles stimulated at 5% radial strain indicated massive amount of cell loss or death. In the 2% radial strain experiments, areas of cell loss and cell death can also be observed, although at a much lower level.

Cytoskeletal protein analysis of the mechanical stimulated samples shows that two-dimensional mechanical strain stimulation promotes the differentiation of the oesophageal smooth muscle cells as indicated by the up-regulation of known differentiation related cytoskeletal proteins. However, this is preceded by a sudden dedifferentiation process at the onset of mechanical stimulation. From the investigation of a known dedifferentiation cytoskeletal marker for cells of the

mesenchymal origin, vimentin is up-regulated throughout the mechanical stimulation experiment, possibly indicating the increased motility of the cells, that is required for the reorientation of cell alignment, in response to the applied stimuli.

In conclusion, cyclic two-dimensional mechanical strain is demonstrated to be a suitable external stimulus to biomimic the physiological state of a normal functional oesophagus thereby inducing cell/tissue remodelling of the oesophageal smooth muscle cells by its cellular reorientation as well as the induction of cellular differentiation closer to the smooth muscle cells found *in vivo*.

6 PROPOSAL FOR FUTURE WORK

In order to enhance the degree of control oesophageal smooth muscle cells through the use of two-dimensional strain mechanical stimulation, investigations should be conducted with the use of different extracellular matrix proteins such as fibronectin, vitronectin, laminin, collagen type I and collagen type IV. Previous research on vascular smooth muscle cells has demonstrated the influences of such matrix proteins to promote phenotype modulation [132, 159-161].

External chemical influences such as the addition of growth factors such as Transforming Growth Factor (TGF- β 1), insulin, Platelet Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF) can also be investigated for their effects on oesophageal smooth muscle cells under the external stimuli of two-dimensional mechanical strain stimulation [132, 146, 162].

Enhancements to the bioreactor system can be made by the integration of live cell imaging capabilities to study the process of migration of cells during two-dimensional strain mechanical stimulation.

For the purpose of tissue engineering three-dimensional cell-scaffold constructs such as smooth muscle cells embedded in collagen gels [150] or/and fibrin gels [163] can also be investigated along with thin elastic porous scaffolds such as salt-leached polyurethane scaffolds [126, 127] or electrospun poly(l-lactide-co-caprolactone) scaffolds [164].

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APPENDIX A
Flow chart of effects of mechanical stimulation on vascular cells

APPENDIX A: Flow chart of effects of mechanical stimulation on vascular cells

APPENDIX B
Detail drawing of two-dimensional strain bioreactor

APPENDIX C

Detail drawing of six-well two-dimensional strain bioreactor

2 X NPT 1/8"-27 (9.16mm)

SCALE 0.500

SCALE 0.500

APPENDIX D
Specification sheets

Introduction to the BASIC Stamps

BASIC Stamp 2

Figure 1.4: BASIC Stamp 2 (Rev. E) (BS2-IC)

Figure 1.5: OEM BASIC Stamp 2 (Rev. A) (27290 or 27291)

The BASIC Stamp 2 is available in the above two physical packages. The BS2-IC (Figure 1.4) uses surface mount components to fit in a small 24-pin DIP package. The OEMBS2 (Figure 1.5) features an easier-to-trace layout meant to aid customers who wish to integrate the BASIC Stamp 2 circuit directly into their design (as a lower-cost solution). The OEMBS2 is available in either an assembled form or a kit form. Both packages are functionally equivalent.

1: Introduction to the BASIC Stamps

Table 1.2: BASIC Stamp 2 Pin Descriptions.

Pin	Name	Description
1	SOUT	Serial Out: connects to PC serial port RX pin (DB9 pin 2 / DB25 pin 3) for programming.
2	SIN	Serial In: connects to PC serial port TX pin (DB9 pin 3 / DB25 pin 2) for programming.
3	ATN	Attention: connects to PC serial port DTR pin (DB9 pin 4 / DB25 pin 20) for programming.
4	VSS	System ground: (same as pin 23) connects to PC serial port GND pin (DB9 pin 5 / DB25 pin 7) for programming.
5-20	P0-P15	General-purpose I/O pins: each can sink 25 mA and source 20 mA. However, the total of all pins should not exceed 50 mA (sink) and 40 mA (source) if using the internal 5-volt regulator. The total per 8-pin groups (P0 – P7 or P8 – 15) should not exceed 50 mA (sink) and 40 mA (source) if using an external 5-volt regulator.
21	VDD	5-volt DC input/output: if an unregulated voltage is applied to the VIN pin, then this pin will output 5 volts. If no voltage is applied to the VIN pin, then a regulated voltage between 4.5V and 5.5V should be applied to this pin.
22	RES	Reset input/output: goes low when power supply is less than approximately 4.2 volts, causing the BASIC Stamp to reset. Can be driven low to force a reset. This pin is internally pulled high and may be left disconnected if not needed. Do not drive high.
23	VSS	System ground: (same as pin 4) connects to power supply's ground (GND) terminal.
24	VIN	Unregulated power in: accepts 5.5 - 15 VDC (6-40 VDC on BS2-IC rev. e), which is then internally regulated to 5 volts. May be left unconnected if 5 volts is applied to the VDD (+5V) pin.

See the "BASIC Stamp Programming Connections" section, below, for more information on the required programming connections between the PC and the BASIC Stamp.

HOST PORT SERIAL CONNECTION

L298

DUAL FULL-BRIDGE DRIVER

- OPERATING SUPPLY VOLTAGE UP TO 46 V
- TOTAL DC CURRENT UP TO 4 A
- LOW SATURATION VOLTAGE
- OVERTEMPERATURE PROTECTION
- LOGICAL "0" INPUT VOLTAGE UP TO 1.5 V (HIGH NOISE IMMUNITY)

DESCRIPTION

The L298 is an integrated monolithic circuit in a 15-lead Multiwatt and PowerSO20 packages. It is a high voltage, high current dual full-bridge driver designed to accept standard TTL logic levels and drive inductive loads such as relays, solenoids, DC and stepping motors. Two enable inputs are provided to enable or disable the device independently of the input signals. The emitters of the lower transistors of each bridge are connected together and the corresponding external terminal can be used for the con-

Multiwatt 15

PowerSO20

ORDERING NUMBERS : L298N (Multiwatt Vert.)
L298HN (Multiwatt Horiz.)
L298P (PowerSO20)

nection of an external sensing resistor. An additional supply input is provided so that the logic works at a lower voltage.

BLOCK DIAGRAM

L298

ABSOLUTE MAXIMUM RATINGS

Symbol	Parameter	Value	Unit
V_S	Power Supply	50	V
V_{SS}	Logic Supply Voltage	7	V
V_I, V_{en}	Input and Enable Voltage	-0.3 to 7	V
I_O	Peak Output Current (each Channel)		
	- Non Repetitive ($t = 100\mu s$)	3	A
	- Repetitive (80% on -20% off; $t_{on} = 10ms$)	2.5	A
	- DC Operation	2	A
V_{sens}	Sensing Voltage	-1 to 2.3	V
P_{tot}	Total Power Dissipation ($T_{case} = 75^\circ C$)	25	W
T_{op}	Junction Operating Temperature	-25 to 130	$^\circ C$
T_{stg}, T_j	Storage and Junction Temperature	-40 to 150	$^\circ C$

PIN CONNECTIONS (top view)

THERMAL DATA

Symbol	Parameter	PowerSO20	Multiwatt15	Unit
$R_{th\ j-case}$	Thermal Resistance Junction-case	Max. -	3	$^\circ C/W$
$R_{th\ j-amb}$	Thermal Resistance Junction-ambient	Max. 13 (*)	35	$^\circ C/W$

(*) Mounted on aluminum substrate

PIN FUNCTIONS (refer to the block diagram)

MW.15	PowerSO	Name	Function
1;15	2;19	Sense A; Sense B	Between this pin and ground is connected the sense resistor to control the current of the load.
2;3	4;5	Out 1; Out 2	Outputs of the Bridge A; the current that flows through the load connected between these two pins is monitored at pin 1.
4	6	V _S	Supply Voltage for the Power Output Stages. A non-inductive 100nF capacitor must be connected between this pin and ground.
5;7	7;9	Input 1; Input 2	TTL Compatible Inputs of the Bridge A.
6;11	8;14	Enable A; Enable B	TTL Compatible Enable Input: the L state disables the bridge A (enable A) and/or the bridge B (enable B).
8	1,10,11,20	GND	Ground.
9	12	V _{SS}	Supply Voltage for the Logic Blocks. A100nF capacitor must be connected between this pin and ground.
10; 12	13;15	Input 3; Input 4	TTL Compatible Inputs of the Bridge B.
13; 14	16;17	Out 3; Out 4	Outputs of the Bridge B. The current that flows through the load connected between these two pins is monitored at pin 15.
–	3;18	N.C.	Not Connected

ELECTRICAL CHARACTERISTICS (V_S = 42V; V_{SS} = 5V, T_j = 25°C; unless otherwise specified)

Symbol	Parameter	Test Conditions	Min.	Typ.	Max.	Unit
V _S	Supply Voltage (pin 4)	Operative Condition	V _{IH} +2.5		46	V
V _{SS}	Logic Supply Voltage (pin 9)		4.5	5	7	V
I _S	Quiescent Supply Current (pin 4)	V _{en} = H; I _L = 0 V _i = L V _i = H		13 50	22 70	mA mA
		V _{en} = L V _i = X			4	mA
I _{SS}	Quiescent Current from V _{SS} (pin 9)	V _{en} = H; I _L = 0 V _i = L V _i = H		24 7	36 12	mA mA
		V _{en} = L V _i = X			6	mA
V _{iL}	Input Low Voltage (pins 5, 7, 10, 12)		–0.3		1.5	V
V _{iH}	Input High Voltage (pins 5, 7, 10, 12)		2.3		V _{SS}	V
I _{iL}	Low Voltage Input Current (pins 5, 7, 10, 12)	V _i = L			–10	μA
I _{iH}	High Voltage Input Current (pins 5, 7, 10, 12)	V _i = H ≤ V _{SS} –0.6V		30	100	μA
V _{en} = L	Enable Low Voltage (pins 6, 11)		–0.3		1.5	V
V _{en} = H	Enable High Voltage (pins 6, 11)		2.3		V _{SS}	V
I _{en} = L	Low Voltage Enable Current (pins 6, 11)	V _{en} = L			–10	μA
I _{en} = H	High Voltage Enable Current (pins 6, 11)	V _{en} = H ≤ V _{SS} –0.6V		30	100	μA
V _{CEsat} (H)	Source Saturation Voltage	I _L = 1A I _L = 2A	0.95	1.35 2	1.7 2.7	V V
V _{CEsat} (L)	Sink Saturation Voltage	I _L = 1A (5) I _L = 2A (5)	0.85	1.2 1.7	1.6 2.3	V V
V _{CEsat}	Total Drop	I _L = 1A (5) I _L = 2A (5)	1.80		3.2 4.9	V V
V _{sens}	Sensing Voltage (pins 1, 15)		–1 (1)		2	V

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ELECTRICAL CHARACTERISTICS (continued)

Symbol	Parameter	Test Conditions	Min.	Typ.	Max.	Unit
$T_1 (V_i)$	Source Current Turn-off Delay	$0.5 V_i$ to $0.9 I_L$ (2); (4)		1.5		μs
$T_2 (V_i)$	Source Current Fall Time	$0.9 I_L$ to $0.1 I_L$ (2); (4)		0.2		μs
$T_3 (V_i)$	Source Current Turn-on Delay	$0.5 V_i$ to $0.1 I_L$ (2); (4)		2		μs
$T_4 (V_i)$	Source Current Rise Time	$0.1 I_L$ to $0.9 I_L$ (2); (4)		0.7		μs
$T_5 (V_i)$	Sink Current Turn-off Delay	$0.5 V_i$ to $0.9 I_L$ (3); (4)		0.7		μs
$T_6 (V_i)$	Sink Current Fall Time	$0.9 I_L$ to $0.1 I_L$ (3); (4)		0.25		μs
$T_7 (V_i)$	Sink Current Turn-on Delay	$0.5 V_i$ to $0.9 I_L$ (3); (4)		1.6		μs
$T_8 (V_i)$	Sink Current Rise Time	$0.1 I_L$ to $0.9 I_L$ (3); (4)		0.2		μs
$f_c (V_i)$	Commutation Frequency	$I_L = 2A$		25	40	KHz
$T_1 (V_{en})$	Source Current Turn-off Delay	$0.5 V_{en}$ to $0.9 I_L$ (2); (4)		3		μs
$T_2 (V_{en})$	Source Current Fall Time	$0.9 I_L$ to $0.1 I_L$ (2); (4)		1		μs
$T_3 (V_{en})$	Source Current Turn-on Delay	$0.5 V_{en}$ to $0.1 I_L$ (2); (4)		0.3		μs
$T_4 (V_{en})$	Source Current Rise Time	$0.1 I_L$ to $0.9 I_L$ (2); (4)		0.4		μs
$T_5 (V_{en})$	Sink Current Turn-off Delay	$0.5 V_{en}$ to $0.9 I_L$ (3); (4)		2.2		μs
$T_6 (V_{en})$	Sink Current Fall Time	$0.9 I_L$ to $0.1 I_L$ (3); (4)		0.35		μs
$T_7 (V_{en})$	Sink Current Turn-on Delay	$0.5 V_{en}$ to $0.9 I_L$ (3); (4)		0.25		μs
$T_8 (V_{en})$	Sink Current Rise Time	$0.1 I_L$ to $0.9 I_L$ (3); (4)		0.1		μs

- 1) 1)Sensing voltage can be $-1 V$ for $t \leq 50 \mu sec$; in steady state $V_{sens} min \geq -0.5 V$.
2) See fig. 2.
3) See fig. 4.
4) The load must be a pure resistor.

Figure 1 : Typical Saturation Voltage vs. Output Current.

Figure 2 : Switching Times Test Circuits.

Note : For INPUT Switching, set EN = H
For ENABLE Switching, set IN = H

Figure 3 : Source Current Delay Times vs. Input or Enable Switching.

Figure 4 : Switching Times Test Circuits.

Note : For INPUT Switching, set EN = H
For ENABLE Switching, set IN = L

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Figure 5 : Sink Current Delay Times vs. Input 0 V Enable Switching.

Figure 6 : Bidirectional DC Motor Control.

Figure 7 : For higher currents, outputs can be paralleled. Take care to parallel channel 1 with channel 4 and channel 2 with channel 3.

APPLICATION INFORMATION (Refer to the block diagram)

1.1. POWER OUTPUT STAGE

The L298 integrates two power output stages (A ; B). The power output stage is a bridge configuration and its outputs can drive an inductive load in common or differential mode, depending on the state of the inputs. The current that flows through the load comes out from the bridge at the sense output : an external resistor (R_{SA} ; R_{SB} .) allows to detect the intensity of this current.

1.2. INPUT STAGE

Each bridge is driven by means of four gates the input of which are $In1$; $In2$; EnA and $In3$; $In4$; EnB . The In inputs set the bridge state when The En input is high ; a low state of the En input inhibits the bridge. All the inputs are TTL compatible.

2. SUGGESTIONS

A non inductive capacitor, usually of 100 nF, must be foreseen between both V_s and V_{ss} , to ground, as near as possible to GND pin. When the large capacitor of the power supply is too far from the IC, a second smaller one must be foreseen near the L298.

The sense resistor, not of a wire wound type, must be grounded near the negative pole of V_s that must be near the GND pin of the I.C.

Each input must be connected to the source of the driving signals by means of a very short path.

Turn-On and Turn-Off : Before to Turn-ON the Supply Voltage and before to Turn it OFF, the Enable input must be driven to the Low state.

3. APPLICATIONS

Fig 6 shows a bidirectional DC motor control Schematic Diagram for which only one bridge is needed. The external bridge of diodes $D1$ to $D4$ is made by four fast recovery elements ($t_{rr} \leq 200$ nsec) that must be chosen of a V_F as low as possible at the worst case of the load current.

The sense output voltage can be used to control the current amplitude by chopping the inputs, or to provide overcurrent protection by switching low the enable input.

The brake function (Fast motor stop) requires that the Absolute Maximum Rating of 2 Amps must never be overcome.

When the repetitive peak current needed from the load is higher than 2 Amps, a paralleled configuration can be chosen (See Fig.7).

An external bridge of diodes are required when inductive loads are driven and when the inputs of the IC are chopped ; Schottky diodes would be preferred.

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This solution can drive until 3 Amps In DC operation and until 3.5 Amps of a repetitive peak current.

On Fig 8 it is shown the driving of a two phase bipolar stepper motor ; the needed signals to drive the inputs of the L298 are generated, in this example, from the IC L297.

Fig 9 shows an example of P.C.B. designed for the application of Fig 8.

Figure 8 : Two Phase Bipolar Stepper Motor Circuit.

This circuit drives bipolar stepper motors with winding currents up to 2 A. The diodes are fast 2 A types.

Fig 10 shows a second two phase bipolar stepper motor control circuit where the current is controlled by the I.C. L6506.

Figure 9 : Suggested Printed Circuit Board Layout for the Circuit of fig. 8 (1:1 scale).

Figure 10 : Two Phase Bipolar Stepper Motor Control Circuit by Using the Current Controller L6506.

L298

DIM.	mm			inch		
	MIN.	TYP.	MAX.	MIN.	TYP.	MAX.
A			5			0.197
B			2.65			0.104
C			1.6			0.063
D		1			0.039	
E	0.49		0.55	0.019		0.022
F	0.66		0.75	0.026		0.030
G	1.02	1.27	1.52	0.040	0.050	0.060
G1	17.53	17.78	18.03	0.690	0.700	0.710
H1	19.6			0.772		
H2			20.2			0.795
L	21.9	22.2	22.5	0.862	0.874	0.886
L1	21.7	22.1	22.5	0.854	0.870	0.886
L2	17.65		18.1	0.695		0.713
L3	17.25	17.5	17.75	0.679	0.689	0.699
L4	10.3	10.7	10.9	0.406	0.421	0.429
L7	2.65		2.9	0.104		0.114
M	4.25	4.55	4.85	0.167	0.179	0.191
M1	4.63	5.08	5.53	0.182	0.200	0.218
S	1.9		2.6	0.075		0.102
S1	1.9		2.6	0.075		0.102
Dia1	3.65		3.85	0.144		0.152

OUTLINE AND
MECHANICAL DATA

Multiwatt15 V

L298

DIM.	mm			inch		
	MIN.	TYP.	MAX.	MIN.	TYP.	MAX.
A			5			0.197
B			2.65			0.104
C			1.6			0.063
E	0.49		0.55	0.019		0.022
F	0.66		0.75	0.026		0.030
G	1.14	1.27	1.4	0.045	0.050	0.055
G1	17.57	17.78	17.91	0.692	0.700	0.705
H1	19.6			0.772		
H2			20.2			0.795
L		20.57			0.810	
L1		18.03			0.710	
L2		2.54			0.100	
L3	17.25	17.5	17.75	0.679	0.689	0.699
L4	10.3	10.7	10.9	0.406	0.421	0.429
L5		5.28			0.208	
L6		2.38			0.094	
L7	2.65		2.9	0.104		0.114
S	1.9		2.6	0.075		0.102
S1	1.9		2.6	0.075		0.102
Dia1	3.65		3.85	0.144		0.152

OUTLINE AND MECHANICAL DATA

Multiwatt15 H

D.C. geared motors with brushes

→ 1.2 Nm 10 and 17 Watts

- A range of D.C. geared motors with solid metal gears
Mechanical rating of gearbox with output shaft stalled : 1.2 Nm
- 10 and 17 Watt motor power
- Available in either 12, 24 or 48 V D.C.
- Gearbox ratios options for 20 to 100 rpm

Specifications

		17 Watts	17 Watts	10 Watts	10 watts
Type		82 802 0	82 802 0	82 812 0	82 812 0
Voltage		12 V	24 V	12 V	24 V
Output speed (rpm)	Ratios (i)				
100	26	•	•	•	•
80	32.5	•	•	•	•
60	130/3	•	•	•	•
38	67.6	•	•	•	•
30	598/7	•	•	•	•
20	130	•	•	•	•
General characteristics					
Motor		82 800 0	82 800 0	82 810 0	82 810 0
Gearbox		81 032 1	81 032 1	81 032 1	81 032 1
Maximum permitted torque from gearmotor under continuous conditions for 10 millions turns (Nm)		1.2	1.2	1.2	1.2
Axial load dynamic (daN)		3.5	3.5	3.5	3.5
Radial load dynamic (daN)		5	5	5	5
Max. output (W)		16.3	17	10.3	9.5
Nominal output (W)		15.7	15.6	9.4	8.7
Gearbox case temperature rise (°C)		44	40	45	46
Weight (g)		670	670	670	670

Product adaptations

- Special supply voltages
- Special cable lengths
- Optional encoder
- Special connectors
- Special output shafts
- Special gearbox ratios
- Special gear wheel material
- Special output bearings
- Special mounting plate

To order, see page 13

Curves

The shaded zone represents the operating range of the geared motor.
The horizontal line marks the maximum torque available in continuous duty cycle for a given life.
For higher torque ratings, service life will be reduced.

Nominal speed and torque curves
82 802 0

Nominal speed and torque curves
82 812 0

Dimensions

82 802 0

- ① 4 holes M4 depth 7.5 mm
- ② 3 holes M5 at 120° depth 7.5 mm
- ③ 2 tags IEC 760 series 4.8 x 0.5 mm
- ④ 7 mm across flats
- ⑤ (shaft pushed-in ←)

82 812 0

- ① 8 holes M4 depth 7.5 mm
- ② 3 holes M5 at 120° depth 7.5 mm
- ③ 2 tags IEC 760 series 4.8 x 0.5 mm
- ④ 7 mm across flats
- ⑤ (shaft pushed-in ←)

MEAN WELL
SWITCHING POWER SUPPLY
ISO-9001 CERTIFIED MANUFACTURER

D-50
SERIES

- LOW COST, HIGH RELIABILITY
- NO OUTPUT CAPACITOR
- WIDE INTERNATIONAL AC INPUT RANGE
- HIGH EFFICIENCY, LOW WORKING TEMPERATURE
- T-SHUT DOWN CIRCUIT, LIMITING AC SURGE CURRENT
- THERMAL PROTECTION CIRCUIT, OVERLOAD PROTECTED
- COMPACT SIZE, LIGHT WEIGHT
- 100% FULL LOAD BURN-IN TEST
- BUILT IN EMI FILTER, LOW RIPPLE NOISE

SPECIFICATION	MODEL	D-50A		D-50B	
		CH1	CH2	CH1	CH2
OUTPUT VOLTAGE		5V	12V	5V	24V
OUTPUT V. TOLERANCE		±2%	+5,-8%	±2%	±8%
OUTPUT RATED CURRENT		6A	2A	6A	1A
OUTPUT CURRENT RANGE		1-6A	0.3-2A	1-6A	0.2-1A
RIPPLE & NOISE		50mVp-p	100mVp-p	50mVp-p	100mVp-p
VOLTAGE REGULATION		±0.5%	±1%	±0.5%	±1%
LOAD REGULATION		±0.5%	±5%	±0.5%	±8%
OUTPUT POWER		54W		54W	
EFFICIENCY		73%		73%	
VOLTAGE ADJ.		CH1:±10,-5%		CH1:±10,-5%	
INPUT VOLTAGE RANGE		85~132VAC / 170~264VAC SELECTED BY SWITCH 47~63Hz; 240~370VDC			
INPUT CURRENT		1.3A/115V 0.65A/230V			
SHUTDOWN CURRENT		COLD START 18A/115V 36A/230V			
NO-LOAD CURRENT		<0.5mA/240VAC			
OVERLOAD PROTECTION		105%~150% TYPE:FOLDBACK CURRENT LIMITING RESET:AUTO RECOVERY			
OVERVOLTAGE PROTECTION		-----			
OVERTEMP. PROTECTION		-----			
TEMP. COEFFICIENT		±0.03% / °C (0~50°C) ON +5V OUTPUT			
POWER, RISE, HOLD UP TIME		200ms, 50ms, 20ms			
SWITCHING FREQUENCY		10~500Hz, 2G 10min./1cycle, PERIOD FOR 60min. EACH AXES			
STAND-BY VOLTAGE		I/P-O/P:1.5KVAC I/P-FG:1.5KVAC O/P-FG:0.5KVAC			
ISOLATION RESISTANCE		I/P-O/P, I/P-FG, O/P-FG:500VDC / 100M Ohms			
WORKING TEMP., HUMIDITY		-10°C~+60°C(REFER TO OUTPUT DERATING CURVE), 20%~90% RH			
STORAGE TEMP., HUMIDITY		-20°C~+85°C, 10%~95% RH			
DIMENSION		159*97*38mm CASE:901			
WEIGHT		0.52Kgs			
SAFETY STANDARDS		MEET UL1012 REQUIREMENT (NOT APPLY FOR THE MARK)			
OTHER STANDARDS		-----			

1. ALL PARAMETERS ARE SPECIFIED AT 230VAC INPUT, RATED LOAD, 25°C 70% RH, AMBIENT.
2. TOLERANCE INCLUDE SET UP TOLERANCE, LINE REGULATION, LOAD REGULATION.
3. RIPPLE & NOISE ARE MEASURED AT 20MHz BY USING A 12" TWISTED PAIR TERMINATED WITH A 0.1uF & 47uF CAPACITOR.
4. LINE REGULATION IS MEASURED FROM LOW LINE TO HIGH LINE AT RATED LOAD.
5. LOAD REGULATION IS MEASURED FROM 20% TO 100% RATED LOAD, AND OTHER OUTPUT AT 60% RATED LOAD.
6. EACH OUTPUT PROVIDE UP TO MAXIMUM CURRENT, BUT TOTAL LOAD CAN NOT EXCEED MAX. OUTPUT POWER.

D-50 SERIES

BLOCK DIAGRAM

$f_{osc} = 24KHz$ I/P-O/P: RATED

OUTPUT DERATING

STATIC CHARACTERISTICS

DIMENSION (mm)

- ☒ CASE : 901
- ☒ TERMINAL PIN NO. ASSIGNMENT
- ☒ PIN 1,2 : AC INPUT
- ☒ PIN 3 : FG $\oplus$
- ☒ PIN 4 : DC OUTPUT +V2
- ☒ PIN 5,6 : DC OUTPUT COM
- ☒ PIN 7 : DC OUTPUT +V1

APPENDIX E
PBASIC source code for actuator control

APPENDIX E: PBASIC source code for actuator control

```
'{$STAMP BS2}
'{$PBASIC 2.0}

'Declaration
  DIRS=$fff 'Pin directions specification
  posn VAR Word
  dir VAR Bit 'Determine movement direction

'initialisation settings
  posn=100 'set initial starting point for position debug
  dir=1 'set initial starting direction to forward
  delay_time_1 CON 2500 'holding time value 1 for PAUSE
  delay_time_2 CON 2500 'holding time value 2 for PAUSE

loop:
  IF IN6=1 THEN again
  LOW 3 ' enable A (switches off L298N half bridge)
  GOTO loop

again:
  IF IN5=1 THEN backward 'sensor 1 contacted
  IF IN4=1 THEN forward 'sensor 2 contacted

move:
  DEBUG "position: ", DEC posn, "dir: ", DEC dir, " sensor1: ", DEC
IN4, "sensor2: ", DEC IN5,CR ' "cycle count: ", DEC cycle,CR
  IF dir=1 THEN move1 'move forward
  IF dir=0 THEN move2 'move backward

move1:
  HIGH 1 'input 1
  LOW 2 'input2
  PWM 3, 255, 128 'PWM output on enable A

  posn=posn+1 'for debug function
  GOTO loop

move2:
  LOW 1 'input 1
  HIGH 2 'input 2
  PWM 3, 255, 128 'PWM output on enable A

  posn=posn-1 'for debug function
  GOTO loop

forward:
  PAUSE delay_time_1
  dir=1
  GOTO move

backward:
  PAUSE delay_time_2
  dir=0
  GOTO move
```

APPENDIX F
Oesophageal smooth muscle cells isolation protocol

APPENDIX F: Oesophageal smooth muscle cells isolation protocol

To isolate oesophageal smooth muscle cells from the porcine oesophageal samples, the following solutions were used. The formulations of the required solutions are listed as follows:

Transport Medium

97.8% Hanks Balanced Salts Solution without Ca^{++} and Mg^{++} (Sigma, St. Louis, Missouri)

2% Antibiotic Antimycotic Solution (AAS) Stabilised (Sigma, St. Louis, Missouri)

0.2% Kanamycin Solution (KS) (Sigma, St. Louis, Missouri)

Primary Culture Medium

77.8% HyQ®DME/High Glucose (DMEM) (Hyclone, Logan, Utah)

20% Fetal Bovine Serum (FBS), origin South America (Hyclone, Logan, Utah, Lot No.: CPC0107)

2% Antibiotic Antimycotic Solution (AAS) Stabilised (Sigma, St. Louis, Missouri)

0.2% Kanamycin Solution (KS) (Sigma, St. Louis, Missouri)

Enzymatic Digestion Solution

5ml Primary Culture Medium

5mg Collagenase Type I, from *Clostridium histolyticum* (Sigma-Aldrich, St. Louis, Missouri)

Elastase Type III Powder

2mg Elastase Type III, from Porcine Pancreas (Sigma, St. Louis, Missouri)

Porcine Oesophageal Smooth Muscle Cell Isolation Protocol

The following procedures were performed for the isolating of oesophageal smooth muscle cells from the porcine oesophageal sample.

1. Porcine oesophageal sample is collected within an hour after the animal was sacrificed.
2. The sample collected is about 20mm in length from the position about 10mm above the lower oesophageal sphincter.
3. The sample is then immersed in 25 ml of transport buffered solution inside a sterile 50ml centrifuge tube and packed in ice during transportation to the laboratory.
4. Upon reaching the laboratory, the sample can be immediately processed or left in a 4°C refrigerator for up to 2 hours before being process.
5. The following procedures are performed inside a Biological Safety Cabinet Class II (Pall Gelman Sciences Corporation, Ann Arbor, Michigan).
6. The sample is removed from the transport medium and placed onto a sterile 100mm diameter Petri dish.
7. The tubular sample is cut longitudinally to form a sheet with a dissecting sissors. The mucosa and submucosa layers are then removed by cutting through the loose connective tissue between the submucosa layer and the muscularis externa. The loose connective tissue on both sides of the muscularis externa is then trimmed away.

APPENDIX F: Oesophageal smooth muscle cells isolation protocol

8. After trimming away the loose connective tissue, the muscularis externa is then rinsed twice with fresh transport medium to wash away any loose connective tissue debris.
9. The muscularis externa is then minced into pieces of about 1 to 2mm³ in size and transferred into a sterile 50ml centrifuge tube.
10. The enzymatic digestion solution is then added to the minced tissue and left to incubate at 37°C for 1 hour.
11. After 1 hour of incubation, 2mg of Elastase Type III (final concentration: 0.4ml/ml) is then added to the enzymatic digestion solution and further incubated for an additional hour.
12. After 2 hours of incubation, the enzymatic digestion solution passed through a 100µm cell strainer (BD Biosciences, Franklin Lake, NJ) and collected in sterile 50ml centrifuge tube. The large pieces of tissues left in the cell strainer are discarded or can be further digested by repeating steps 8 to 10.
13. The sieved enzymatic digestion solution containing single cells are then centrifuged at 1000 RCF for 5 minutes. The resulting cell pellet is then collected and the supernatant is discarded.
14. The cell pellet is then resuspended in 1ml of primary culture medium by vigorous pipetting using a 1000µl micropipette to form single cell suspension.
15. 100µl of single cell suspension is then transferred to 60mm diameter tissue culture plates with 4ml of primary culture medium.
16. The tissue culture plates are then incubated in a humidified 5% CO₂ incubator set at 37°C and exhausted primary culture medium was exchanged with fresh primary culture medium every 2 to 3 days until the cells are grown to confluency.
17. Upon reaching confluency, the cells would be sub-cultured as according to the protocol found in Appendix G.

APPENDIX G
Oesophageal smooth muscle cells sub-cultivation protocol

APPENDIX G: Oesophageal smooth muscle cells sub-cultivation protocol

When the oesophageal smooth muscle cells have grown to form a confluent monolayer within a tissue culture container, it is necessary to dissociate them from the existing tissue culture container and divide them among new containers for the cells to continue proliferating.

The formulations of the required solutions are listed as follows:

Cell Culture Medium

88.8% HyQ®DME/High Glucose (DMEM) (Hyclone, Logan, Utah)

10% Fetal Bovine Serum (FBS), origin South America (Hyclone, Logan, Utah, Lot No.: CPC0107)

1% Antibiotic Antimycotic Solution (AAS) Stabilised (Sigma, St. Louis, Missouri)

0.2% Kanamycin Solution (KS) (Sigma, St. Louis, Missouri)

Buffered Rinsing Solution

1× Phosphate Buffered Saline (PBS) without Ca^{++} and Mg^{++} , pH 7.4 (Sigma, St. Louis, Missouri)

Cell Dissociating Agent

0.25% Trypsin-EDTA (Sigma, St. Louis, Missouri)

Porcine Oesophageal Smooth Muscle Cells Sub-cultivation Protocol

1. Exhausted cell culture medium is removed from the tissue culture container and discarded.
2. Sterile 1× PBS is then dispensed into the tissue culture container gently to rinse the confluent monolayer to wash away any cell debris and discarded. This procedure is performed twice.
3. 2ml of 0.25% Trypsin-EDTA solution is added to the confluent monolayer and incubated at 37°C for 3 minutes. As a rule of thumb, 2ml of Trypsin-EDTA solution is added for every 25 cm² of confluent cell monolayer.
4. After incubation for 3 minutes, the tissue culture container is observed under the microscope for detachment of cell from the culture surface. Tap the sides of the tissue culture container gently to aid the cell from detaching from the culture surface. If cells are still attached, incubate the tissue culture container at 37°C for a further 2 minutes.
5. Once most of the cells have detached from the culture surface (>95%), add an equal amount of cell culture medium into the tissue culture container to neutralise the Trypsin.
6. Aspirate the cell suspension from the tissue culture container and place it in a sterile centrifuge tube.
7. Centrifuge the cell suspension at 1000 RCF (rotational centrifugal force) RCF for 5 minutes to form a cell pellet at the bottom of the centrifuge tube.
8. After centrifugation, gently remove the supernatant from the tube and discard.
9. Add 1ml of cell culture medium to the centrifuge tube and resuspend the cell pellet into a single cell suspension by vigorous pipetting with a 1000µl micropipette.
10. The cell suspension is then divided into 5 parts and one part is dispensed into each new tissue culture containers containing fresh cell culture medium.

APPENDIX G: Oesophageal smooth muscle cells sub-cultivation protocol

11. The new tissue culture containers seeded with cell are then incubated inside a humidified 5% CO₂ incubator set at 37°C for the cells to grow. The cell culture medium would have to be replaced with fresh cell culture medium every 2 to 3 days.
12. If cell number is required to be determined, a Neubauer improved bright-line haemocytometer (Paul Marienfeld GmbH & Co. KG, Germany) may be used. A sample of the cell suspension is transferred into a microcentrifuge tube and diluted appropriately with an equal amount of 0.4% Trypan Blue solution (Sigma, St. Louis, Missouri). 10µl of the diluted cell suspension is then dispensed under the glass cover slip of the haemocytometer and counted under a microscope.

APPENDIX H
**Immunocytochemical staining of oesophageal smooth muscle
cells**

APPENDIX H: Immunocytochemical staining of oesophageal smooth muscle cells

In order to positively identify the cells isolated from the porcine oesophageal sample, immunocytochemical staining is performed. Immunocytochemical staining utilises the highly specific antibody-antigen interaction between two proteins. For the purpose of characterisation of our isolated cells, smooth muscle cell specific cytoskeletal markers such as smooth muscle α -actin, desmin and smooth muscle myosin heavy chain are stained for using their respective antibodies.

The formulations of the required solutions are listed below:

Antibody Diluent Solution

50mg Bovine Serum Albumin (Sigma, St. Louis, Missouri)

50ml Phosphate Buffered Saline, pH 7.4 (Sigma, St. Louis, Missouri)

Blocking Medium

4% Goat Serum (Dako, Glostrup, Denmark)

96% Antibody Diluent Solution

Antibody Diluent Solution with 2% Goat Serum

10mg Bovine Serum Albumin (Sigma, St. Louis, Missouri)

10ml Phosphate Buffered Saline, pH 7.4 (Sigma, St. Louis, Missouri)

0.2ml Goat Serum (Dako, Glostrup, Denmark)

Primary Antibody Solution (100 $\times$ dilution)

1% Monoclonal Mouse anti-Smooth Muscle α -actin (Clone 1A4) (Dako, Glostrup, Denmark)

OR

1% Monoclonal Mouse anti-Desmin (Chemicon International, Inc., Temecula, CA)

OR

1% Monoclonal Mouse anti-Smooth Muscle Myosin Heavy Chain (SMMHC) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)

AND

99% Antibody Diluent Solution 2% Goat Serum

Negative Control Solution for Primary Antibody (1000 $\times$ dilution)

0.1% Negative Control mouse IgG1 (Dako, Glostrup, Denmark)

99.9% Antibody Diluent Solution 2% Goat Serum

Secondary Antibody Solution (100 $\times$ dilution)

1% Biotinylated Polyclonal Goat Anti-Mouse Immunoglobulins (Dako, Glostrup, Denmark)

99% Antibody Diluent Solution 2% Goat Serum

Tertiary Antibody Solution (50 $\times$ dilution)

2% Streptavidin, TEXAS RED® conjugate (Oncogene Research Products, La Jolla, CA)

OR

2% Streptavidin, FITC conjugate (Dako, Glostrup, Denmark)

98% Antibody Diluent Solution 2% Goat Serum

DAPI Solution

APPENDIX H: Immunocytochemical staining of oesophageal smooth muscle cells

4',6-Diamidino-2-phenylindole dihydrochloride (Sigma, St. Louis Missouri)

Immunocytochemical staining protocol for Oesophageal Smooth Muscle Cells

1. Cells isolated from the porcine oesophageal samples are seeded onto glass cover slips coated with Collagen Type I and incubated overnight at 37°C.
2. Before fixing the attached cells, the glass cover slips are rinsed twice with 1× PBS to remove cell debris and unattached cells.
3. 100% cold Methanol (-20°C) is dispensed onto the glass cover slips and kept in a -20°C freezer for 20 minutes. After 20 minutes, the methanol is removed and the cells are rinsed 3 times in 1× PBS.
4. The blocking medium is then dispensed onto the glass cover
5. The primary antibody solution is then dispensed onto the glass cover slip and incubated for 2 hours at room temperature. For the case of the negative control for the primary antibody, the negative control solution is added in place of the primary antibody solution. After incubation, the primary antibody solution or the negative control solution is removed and the glass cover slips are rinsed 3 times with 1× PBS.
6. The secondary antibody solution is then dispensed to the glass cover slips and incubated for 2 hours at room temperature. After incubation, the secondary antibody solution is removed and the glass cover slips are rinsed 3 times with 1× PBS.
7. The tertiary antibody solution is then dispensed to the glass cover slips and incubated for 2 hours at room temperature. The glass cover slips would have to be kept in the dark from this point onwards to minimise photobleaching.
8. DAPI solution is dispensed onto the glass cover slip and is removed after 1 minute of incubation at room temperature.
9. To mount the glass cover slip, one drop of Dako® Fluorescent Mounting Medium is placed onto a glass slide and the glass cover slip is inverted and placed onto the mounting medium.

With the immunocytochemical staining of isolated oesophageal smooth muscles cells completed, the cells can then be immediately observed under a fluorescence microscope or wrapped in aluminium foil to protect from light and stored away for future use.

APPENDIX I

Protein separation (SDS-PAGE) and immunoblotting protocol

APENDIX I: Protein separation (SDS-PAGE) and immunoblotting protocol

Cytoskeletal protein analysis can be performed on porcine oesophageal smooth muscle cells through the use of antibody-antigen interactions. Before cytoskeletal protein analysis can be carried out, the cytoskeletal proteins would have to be first extracted from the cells. Below is the protocol for extraction of cytoskeletal proteins using cell lysis buffer. The cell lysis buffer used in this protocol is M-PER® Mammalian Protein Extraction Reagent (Pierce, Rockford, IL)

Protocol for extraction of cytoskeletal protein

1. Cell culture medium is removed from the scaffold/bioreactor actuation membrane.
2. Oesophageal smooth muscle cells are rinsed in 1× PBS.
3. 200µl of M-PER® Mammalian Protein Extraction Reagent is added to the cells and incubated for 2 minutes at room temperature.
4. The cell lysis buffer is then collected back in a microcentrifuge tube and centrifuged for 8 minutes at 14,000 RCF.
5. The supernatant is then transferred to a new centrifuge tube and frozen at -86°C for further processing.

After the extraction of cytoskeletal protein from the oesophageal smooth muscle cells, the protein concentration of the cell lysate would have to be first determined. The determination of protein concentration is required for the equal amount of protein loaded for each cell lysate sample to be separated by gel electrophoresis. Quick Start Bradford 1× Dye Reagent (Bio-Rad Laboratories, Hercules, CA) is used for the colorimetric assay and Bovine Serum Albumin Protein Standard (Sigma, St. Louis, Missouri) is used for comparison and quantification of the cell lysate samples.

Protocol for Protein Quantification by Bradford Protein Assay

1. Thaw cell lysate samples to room temperature.
2. Add 10µl of cell lysate sample to 990µl of Quick Start Bradford 1× Dye Reagent in a microcentrifuge tube. Repeat for each cell lysate sample.
3. Incubate cell lysate and dye reagent mixture at room temperature for 5 minutes.
4. Dispense cell lysate and dye reagent 150µl of mixture into 96 well microplate.
5. Obtain light absorbance reading at 595nm.
6. To obtain a protein standard plot, dilute BSA Protein Standard by half 4 times to obtain the protein concentrations of 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml and 0.0625mg/ml. Deionised water is used as the protein standard for 0mg/ml.
7. Repeat steps 2 to 5 for each of the protein standards to obtain a calibration plot used to quantify the protein concentration for each of the cell lysate sample.

After the protein concentration of the cell lysate samples are quantified, the samples are ready to be separated by SDS-PAGE. Separating polyacrylamide gels are prepared at concentrations of 7.5% and 10% depending on the molecular weight of the cytoskeletal protein investigated. The stacking polyacrylamide gel used is 4%. A larger acrylamide concentration would give rise to a smaller pore size. The polyacrylamide gels are prepared using the formulations listed below.

APENDIX I: Protein separation (SDS-PAGE) and immunoblotting protocol

Percent Gel	Deionised Water (ml)	30% Acrylamide (ml)	Gel Buffer (ml)	10% SDS (ml)	10% APS (μl)	TEMED (μl)
4%	6.1	1.3	2.5	0.1	50	10
7.5%	4.9	2.5	2.5	0.1	50	5
10%	4.1	3.3	2.5	0.1	50	5

Protocol for Protein Separation by SDS-PAGE

1. Add 15μl of cell lysate sample to 15μl of Laemmli Sample Buffer for each sample to be separated.
2. Heat the mixture at 95°C for 4 minutes.
3. From the protein concentration determined previously, load 10μg of cell lysate protein into the wells of the polyacrylamide gel.
4. Separate the protein by running the electrophoresis at 100 Volts for about 70 minutes.
5. After completion of the protein separation, the polyacrylamide gels are removed for further processing.

In order to detect the cytoskeletal protein that has been separated by SDS-PAGE, the proteins would have to be transferred from the polyacrylamide gels onto a nitrocellulose membrane using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). The proteins are transferred at 25 Volts for 12 hours. After transferring the proteins, the nitrocellulose membrane would be stained with antibodies to determine its presence. The following reagents that are required in the procedure are listed below:

Washing Buffer

50ml 10× Phosphate Buffered Saline, pH 7.4 (Sigma, St. Louis, Missouri)
 2.5ml 20% (v/v) Tween-20 (Aldrich, St. Louis, Missouri)
 447.5ml deionised H₂O

Blocking Buffer

100mg Bovine Serum Albumin (Sigma, St. Louis, Missouri)
 10ml Washing Buffer

Primary Antibodies

Monoclonal Mouse Anti-Human Smooth Muscle Actin (Clone 1A4) (Dako, Glostrup, Denmark)
 Mouse Anti-Desmin Monoclonal Antibody (Chemicon International, Inc., Temecula, CA)
 Polyclonal Goat Anti-Human Calponin 1 (Clone N15) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)
 Monoclonal Mouse Anti-Rat Smooth Muscle Myosin Heavy Chain Clone (G-4) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)
 Monoclonal Mouse Anti-Porcine Vimentin (Clone V9) (AbD Serotech, Oxford, UK)

APENDIX I: Protein separation (SDS-PAGE) and immunoblotting protocol

Secondary Antibodies

Anti-mouse IgG, Horseradish Peroxidase linked whole antibody (from sheep)
(Amersham Biosciences, Buckinghamshire, UK)

Mouse Anti-Goat IgG-HRP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)

Protocol for Immunoblotting (Western Blot)

1. After completion of the transfer of the proteins from the polyacrylamide gel from the nitrocellulose membrane, the nitrocellulose membrane is washed 3 times in 1× PBS with 0.1% Tween-20 (PBST) for 15 minutes each.
2. After washing, the membrane is incubated with a blocking solution consisting of 1% BSA in PBST for one hour at room temperature on an orbital shaker.
3. The primary antibody is added directly to the blocking solution at a dilution of 1:1000 and incubated for another hour at room temperature.
4. After incubation with the primary antibody, the membrane is washed 3 times again with PBST for 15 minutes each.
5. The membrane is then incubated with the secondary antibody which is diluted in PBST at a dilution factor of 1:2000 for 1 hour at room temperature.
6. After incubation with the secondary antibody, the membrane is washed 3 times again with PBST for 15 minutes each.
7. ECL Detection Reagent 1 and 2 is mixed in a ratio of 1:1 and dispensed onto the membrane for one minute.
8. ECL Hyperfilm is exposed to the membrane for one minute to detect the chemiluminescent signal.
9. The exposed ECL Hyperfilm is then developed in Kodak GBX/Developer for 2 minutes.
10. The ECL Hyperfilm is then rinsed in tap water to remove the developer solution.
11. The ECL Hyperfilm is then fixed in Kodak GBX/Fixer for 2 minutes.
12. The ECL Hyperfilm is then washed with large amounts of tap water and air dried.

Note: Steps 8 to Step 11 requires a photographic dark room.

After the ECL Hyperfilm have been air dried, the film is put into a Model GS-800TM Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA). The separated bands at the correct weights are then boxed up and measured using Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA) to obtain the relative intensity of the cytoskeletal protein expression.