# DEVELOPMENT OF MICRO FLUIDIC BASED DEVICES FOR STUDYING TUMOUR BIOLOGY AND EVALUATING TREATMENT RESPONSE IN HEAD AND NECK CANCER BIOPSIES

being a Thesis submitted for the Degree of Doctorate of Medicine

in the University of Hull

by

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**October 2011**

## Acknowledgements

Firstly to my supervisors: Professor Greenman, whose enthusiasm was endless, knowledge limitless, patience tireless and supply of green ink seemingly bottomless. To Professor Stafford who guided me through applying for funding, tissue collection and general diplomacy, and whose ever open door provided clinical and pastoral support.

Without the support of my sponsors, the Royal College of Surgeons of England, this work would certainly not be possible. Their generosity, not only financial but also extending to research courses, library facilities and continuing support was remarkable and I am forever indebted. I also thank the Henry Chatterton fund whose contribution to consumables was gratefully received.

To the other members of the Head and Neck research team and University of Hull: to Ann Lowry for her expert teaching on tissue sectioning, to Vicky and Anna, for your teaching and patience, and to Sam D for your company and forever advising me how to get out of whatever pickle I had managed to get myself into. A special thank you to Sam H, 'the guru of micro fluidics' whose perseverance and attention to detail will forever astound me, and without whom I would have fallen at the first hurdle.

My friends and family for all of their support, and putting up with me through times of stress yet again, I promise this will be it now (well until the next time). Finally to my Mum, my inspiration behind everything, sadly missed.

## Thesis Associated Publications

**Development of micro fluidic-based analytical methodology for studying the effects of chemotherapy agents on cancer tissue**

Sylvester DC, Hattersley SM, Stafford ND, Haswell SJ, Greenman J

Submitted to Current Analytical Chemistry

**An innovative explant tissue model using micro fluidics for the comparison of tumour tissue responses to chemotherapy regimens**

Hattersley SM, Sylvester DC, Dyer CE, Stafford ND, Haswell SJ, Greenman J

Submitted to Annals of Biomedical Engineering

**Micro fluidic devices for evaluating HNSCC tumor biology**

Sylvester DC, M Hattersley SM, Dyer CE, Stafford ND, Haswell SJ, Greenman J

Otolaryngology - Head and Neck Surgery

August 2010 Vol. 143, Issue 2, Supplement 2, Page P166

## Thesis Associated Presentations

**Development of micro fluidic based devices for studying tumour biology and evaluating treatment response in head and neck cancer biopsies (Poster)**

Sylvester DC, M Hattersley SM, Dyer CE, Stafford ND, Haswell SJ, Greenman J

The 14th International Conference on Miniaturized Systems for Chemistry and Life Sciences, µTAS 2010 Conference, Groningen, The Netherlands, 2010

**Micro fluidic devices for evaluating head and neck tumour biology (Poster)**

Sylvester DC, M Hattersley SM, Stafford ND, Haswell SJ, Greenman J

American Academy of Otolaryngology — Head and Neck Surgery Foundation's Annual Meeting, Boston, US, 2010

**Microfluidic based devices for the evaluation of chemotherapy response in head and neck cancer biopsies (Poster)**

Sylvester DC, M Hattersley SM, Stafford ND, Haswell SJ, Greenman J

Otorhinolaryngology Research Society, Coventry, UK, 2010

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## Abbreviations

| | |
|---|---|
| **5FU** | 5-fluorouracil |
| **7-EC** | 7-ethoxycoumarin |
| **AJCC** | The American Joint Committee on Cancer |
| **ATP** | Adenosine Triphosphate |
| **AUC** | Area Under the Curve |
| **BCNU** | 1,3-bis(2-chloroethyl)-l-nitrosourea |
| **CAD** | Computer Assisted Design |
| **CDCA** | Chenodeoxycholic Acid |
| **CDK** | Cyclin Dependent Kinase |
| **CEA** | Carcinoembryonic Antigen |
| **Cmax** | Mean Observed Peak Concentration |
| **CTC** | Circulating Tumour Cell |
| **CTL/LAK** | Cytotoxic T lymphocyte/human Lymphokine Activated Killer cells |
| **DC** | Dendritic Cells |
| **DNA** | Deoxyribonucleic Acid |
| **EBV** | Epstein-Barr Virus |
| **EC** | Electron Coupling Reagent |
| **ECM** | Extracellular Matrix |
| **EGF** | Epidermal Growth Factor |
| **EGFR** | Epidermal Growth Factor Receptor |
| **ELISA** | Enzyme-linked Immunosorbent Assay |
| **EMD 72000** | Humanised anti-EGFR Monoclonal Antibody Matuzumab |
| **EORTC** | European Organisation for Research and Treatment of Cancer |
| **FAK** | Focal Adhesion Kinase |
| **FdUMP** | Fluorodeoxyuridine Monophosphate |
| **FGF** | Fibroblast Growth factor |
| **GFP** | Green Fluorescent Protein |
| **H&E** | Haematoxylin and Eosin |

| | |
|---|---|
| **HER** | Human Epidermal Growth Factor Receptor |
| **HIV** | Human Immunodeficiency Virus |
| **HMG** | High Mobility Group family proteins |
| **HNSCC** | Head and Neck Squamous Cell Carcinoma |
| **HPV** | Human Papilloma Virus |
| **HUVEC** | Human Umbilical Vein Endothelial Cells |
| **IC** | Intracellular |
| **IHC** | Immunohistochemistry |
| **IMRT** | Intensity Modulated Radio Therapy |
| **IUCC** | The Union for International Cancer Control |
| **LDH** | Lactate Dehydrogenase |
| **LIF** | Leukaemia Inhibitory Factor |
| **LMP** | Latent Membrane Protein |
| **MAPK** | Mitogen Activated Protein Kinase |
| **MIBG** | Meta-iodobenzylguanidine |
| **MIF-1α** | Macrophage Migration Inhibitory Factor 1-alpha |
| **MMP-8** | Matrix Metalloproteinase-8 |
| **mRNA** | messenger Deoxyribonucleic Acid |
| **MTS** | Multicellular Tumour Spheroids |
| **MTT** | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| **NAD+/NADH** | Nicotinamide Adenine Dinucleotide |
| **NPC** | Nasopharyngeal Carcinoma |
| **P-gp** | Permeability glycoprotein |
| **PBMC** | Peripheral Blood Mononuclear Cell |
| **PCNA** | Proliferating Cell Nuclear Antigen |
| **PCR** | Polymerase Chain Reaction |
| **PD** | Pharmacodynamics |
| **PDGF** | Platelet Derived Growth Factor |
| **PDMS** | Poly(dimethylsiloxane) |
| **PI3K** | Phosphatidylinositol 3-kinase |
| **PK** | Pharmacokinetics |

| | |
|---|---|
| **POCT** | Point of Care testing |
| **pRb** | Retinoblastoma protein |
| **RDTs** | Rapid Diagnostic Tests |
| **RR** | Relative Risk |
| **RS** | Mitochondrial Succinate-tetrazolium-reductase System |
| **SAV** | Surface Area to Volume ratios |
| **SCC** | Squamous Cell Carcinoma |
| **STAT** | Signal Transducers and Activators of Transcription |
| **TAA** | Tumour-associated Antigen |
| **SAV** | Surface Area to Volume Ratio |
| **TANIS** | The Tumour and Node Interger Score System |
| **TGF-α/β** | Transforming Growth Factor α/β |
| **TNM** | Tumour Node Metastases |
| **TPF** | Taxotere, Cisplatin and 5FU |
| **UADT** | Upper Aero Digestive Tract |
| **VEGF** | Vascular Endothelial Growth Factor |
| **WHO** | World Health Organisation |
| **WST-1** | Water Soluble Tetrazolium, 4-[3-[4 - Iodophenyl] - 2 - 4 (4 nitrophenyl)- 2H- 5-tetrazolio-1, 3-benzene disulfonate |
| **XTT** | 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide |
| **µCCA** | Microscale Cell Culture Analogue |

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## Abstract

Head and Neck Squamous Cell Carcinoma (HNSCC) presents particular challenges to both the researcher and the clinician. Encompassing a group of tumours with distinct epidemiological and oncological behaviour, each sub site differs greatly in its management and prognosis. However, due to insufficient tissue quantities and culture techniques, research studies commonly group together different sub sites, limiting our understanding of the biological behaviour and treatment response of tumours from distinct sites.

Micro fluidics relates to the science of systems that process or manipulate small amounts of fluids within micro channels. When applied in biology, the technology enables the production of simple, robust and highly versatile systems for studying cells and tissues. The aim of the work in this thesis was to maintain small biopsies of tumour in a physiological state, more comparable to the *in vivo* environment than traditional tissue culture techniques, for up to 9 days. This recreation of the 'tumour microenvironment' *in vitro* provided a platform for the testing of chemotherapy agents and analysing individual tumour behaviour.

Initial optimisation studies were performed to demonstrate tissue viability within this novel culture method. Based on LDH excretion as a marker of cell death and WST-1 metabolism as a marker of viability, tumour and nodal biopsies from a variety of sub sites remained viable within the device until the addition of cell lysis buffer at 68 h. Histo-architectural examination of tissue incubated within the device for 96 h demonstrated that original tissue structure is largely maintained. In addition, comparison of viability between fresh and frozen tissues showed little difference, thus the clinical applicability of the technique was significantly enhanced, as biopsies could be collected and stored prior to use at a later date.

Using clinically relevant combinations of chemotherapy drugs, nodal biopsies (n=50 micro chips from n=2 tumours with all experiments duplicated) were interrogated with cisplatin, 5-Fluorouracil and docetaxel within the micro device for up to 9 days. The addition of each chemotherapeutic agent resulted in increased cell death compared to control, with a synergistic effect seen when agents were given in combination; results in agreement with clinical trial data.

This study demonstrates a robust and reproducible system for the maintenance and 'interrogation' of individual tumour biopsies. The innovative model provides a new platform for testing individual patient responses to chemotherapy, paving the way for 'personalised' treatment regimens.

# 1 Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) accounted for around 3% of all cancers in the UK in 2007 (http://info.cancerresearchuk.org/cancerstats/) but represents the sixth most common cancer worldwide (Ferlay et al., 2010). The condition encompasses malignancies of the oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx. HNSCC is a solid tumour with remarkable ability to evade normal immune surveillance and suppress any elicited immune response. Such tumours often cause high morbidity due to their location, both through loss of function and disfigurement. Current treatments commonly contribute to morbidity; extensive surgical resection can adversely affect speech and swallowing even when local cure is achieved, and the complications of radiotherapy such as xerostomia are often life-long (Gleeson and Scott-Brown, 2008).

Treatment planning options for optimal outcome are becoming increasingly challenging as more and more single and multiple treatment modality regimes become available. New concepts such as induction chemotherapy, integration of targeted therapies, concurrent and sequential chemo-radiation strategies offer an alternative to the traditional definitive surgical procedures (Choong and Vokes, 2008). Predicting outcome is particularly difficult in HNSCC; heterogeneity of tumour response, impaired late functional outcome and increased late toxicity of chemotherapy regimens (especially if simultaneously applied with radiation) all influence treatment response. Retrospectively looking at 'non-responders' to chemo-radiotherapy highlights those for whom primary surgery is likely to have achieved better results. The inability to predict this information beforehand, however, means that some patients will unnecessarily suffer the side effects of chemo-radiotherapy and in addition, this time delay means that the tumour is more advanced when surgery is performed. Having a platform to test therapies on an individual tumour, before they are given to the patient, would mean that the most appropriate modality or chemotherapy regimen is given as first choice.

Micro fluidics relates to the science of systems that process or manipulate small amounts of fluids within micro channels, enabling the production of simple,

reproducible and highly versatile biological tissue systems. The application of micro fluidic devices to the study of tumour biology and treatment response has the potential to revolutionise the way we study not just HNSCC but all solid tumour types.

How well traditional cell based tumour cultures represent the *in vivo* behaviour of a tumour has long been questioned. Tumours exist not just as groups of identical cells, but as part of a complex cellular body, dependant on interactions with normal cells and supporting proteins. Not only is the validity of *in vitro* studies questionable, the length of time taken to culture cells and investigate treatment response means that a real time personalised treatment model is unobtainable. The application of micro fluidic technology serves to mimic *in vivo* tumour conditions whilst providing a practical analytical model for assessing individual tumour response to treatment.

## 1.1 Anatomy and Histology of HNSCC

Head and Neck cancer encompasses a wide range of tumours of anatomical and histological distinction. Although 90% of tumours are of the squamous cell type (Curado and Hashibe, 2009), being derived from the upper-aero digestive tract (UADT), other tumour types derived from skin, salivary glands and the thyroid gland, are, less commonly found. The current work has focused on tumours of squamous cell type, predominantly collected from the larynx, oropharynx, nasopharynx and hypopharynx and associated lymph nodes, reflecting their clinical prevalence and therefore these sites will be briefly described in turn.

The head and neck can be divided into anatomically defined regions, namely the nasal cavity and paranasal sinuses, oral cavity, pharynx (further divided into nasopharynx, oropharynx and hypopharynx) and larynx (Figure 1.1). Approximately 60-70% of head and neck tumours are found within the oral cavity and larynx with tumours of the nasal cavity and paranasal sinuses (3%) being relatively rare (Gleeson and Scott-Brown, 2008).

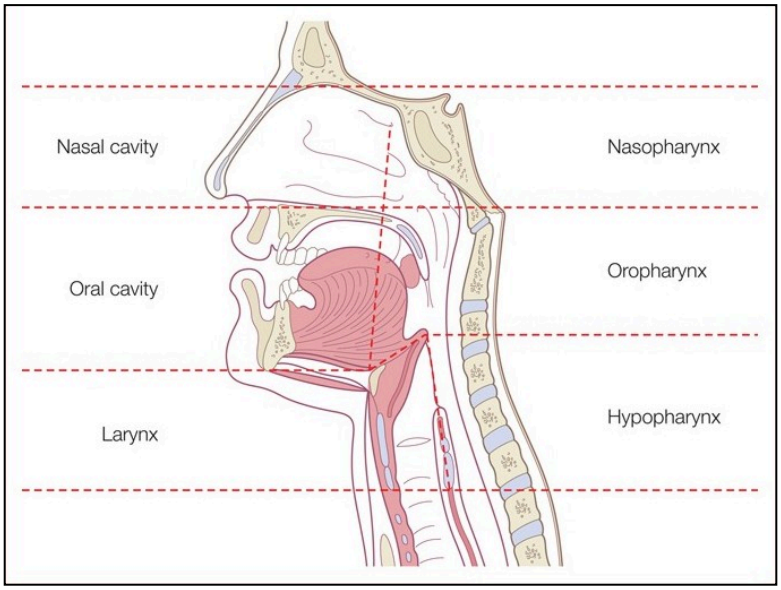



Figure 1.1 Anatomical divisions of the head and neck (Gleeson and Scott-Brown, 2008).

### 1.1.1 The Lymph Nodes of the Neck

For HNSCC, lymphatic spread is a key feature with approximately 40% of patients demonstrating evidence of nodal involvement at presentation as detected clinically or radiologically (Genden et al., 2003). Up to a further 20% of patients presumed 'node-negative' have evidence of micro-metastasis after histopathological evaluation (Ferlito et al., 2001). Given that the presence of lymph node metastasis is associated with a 50% reduction in survival rates on average (Wenzel et al., 2004), predicting lymphatic spread is important in clinical treatment planning. The likely sites of lymph node metastasis for each anatomical site of the head and neck were mapped by a key study by Lindberg et al. (1972) and based on these findings, the descriptive neck levels above (originally five, with a further two levels being added since (Robbins et al., 2008) were described by surgeons at the Memorial Sloan-Kettering Cancer Centre in 1981, thus standardising nomenclature in neck dissection operations.

The lymph node levels, described by their groupings and anatomical locations are summarised as follows (Robbins et al., 2008) and depicted in Figure 1.2:

- Level I, submental and submandibular lymph nodes
- Level II, cervical jugular chain nodes above the level of the hyoid. Further divided into IIa and IIb by their relationship to the accessory nerve
- Level III, cervical jugular chain nodes from the level of the hyoid to the level of the cricoid
- Level IV, cervical jugular chain nodes from the level of the cricoid to the suprasternal notch
- Level V, posterior triangle lymph nodes. Can be further divided into Va and Vb as to whether they are above or below the omohyoid muscle
- Level VI, central compartment nodes
- Level VII, superior mediastinal nodes

Between 100 and 200 lymph nodes are located within each side of the neck, joined together by a complex communication system broadly divided into a superficial and deep network, with lymph draining first into the superficial network

from the UADT and then into the deep before joining the venous system at the thoracic inlet.

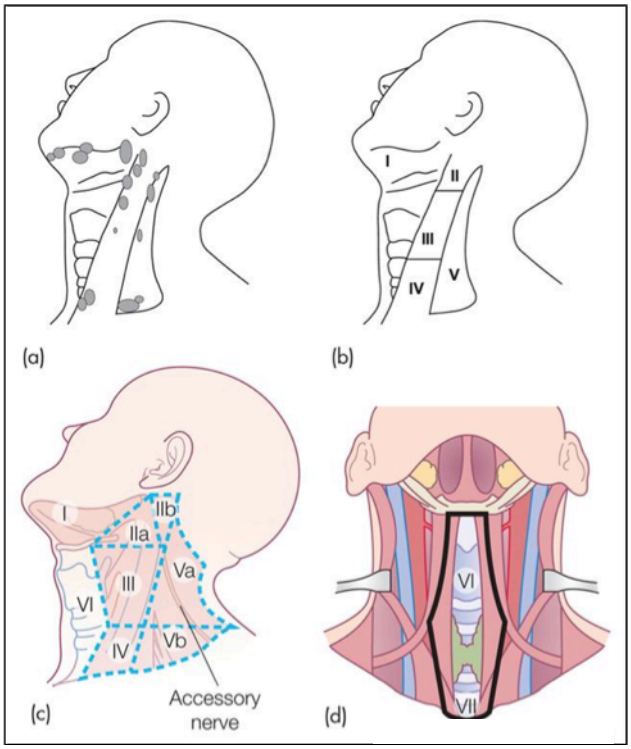



Figure 1.2 The lymph nodes of the neck, a) the anatomical distribution of the lymph nodes of the neck, b) the Memorial Sloane-Kettering levels I-V as described in 1981, c), d) the more recently added divisions and levels I-VII (Gleeson and Scott-Brown, 2008) Adapted from (Bailey, 2001).

### 1.1.2 The Larynx

The larynx serves to provide voice and divide the respiratory tract from the digestive tract. It consists of a cartilaginous framework connected by ligaments, membranes and muscles covered by respiratory and stratified mucosal epithelium. Arterial blood supply is from the external carotid artery and the thyrocervical trunk via the superior and inferior thyroid arteries. Venous drainage is to the internal jugular vein via the superior and inferior thyroid veins. Innervation of the muscles of the larynx is from the recurrent laryngeal branch of the vagus nerve except for cricothyroid which is innervated by the external laryngeal nerve (Last and Sinnatamby, 1999).

The larynx can be divided into three parts: the supraglottis (tip of the epiglottis superiorly to the under surface of the false cords inferiorly), glottis (from the ventricle between the true and false cords to 0.5cm below the free edge of the true cords) and subglottis (the inferior extent of the glottis to the inferior border of the cricoid cartilage) as shown in figure 1.3.

The supraglottis and glottis are composed of the following:

- Supraglottis - suprahyoid epiglottis (tip, lingual and laryngeal surfaces), aryepiglottic fold, arytenoid, infrahyoid epiglottis, false cords
- Glottis – vocal cords, anterior commissure, posterior commisure

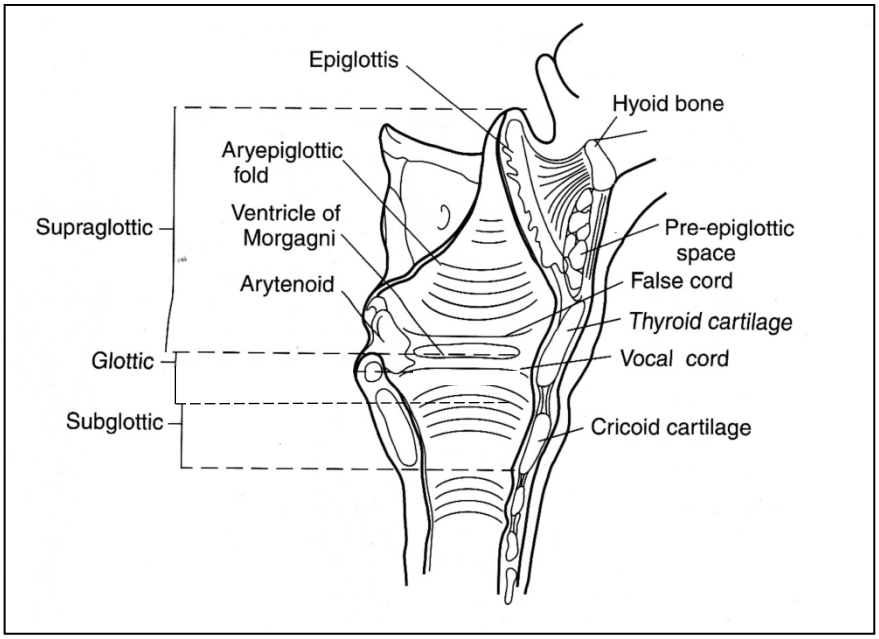



Figure 1.3 The anatomy of the larynx; supraglottis, glottis and subglottis (Gleeson and Scott-Brown, 2008) Adapted from (Bailey, 2001).

Between 30 and 40% of laryngeal cancers arise from the supraglottic area, the most common site being the infrahyoid epiglottis. The glottis is the most common site for laryngeal cancer with 50-70% of tumours located in this area. Tumours in this area can spread superficially across the anterior commisure (the anterior apex of the vocal cords) onto the opposite cord or into adjacent sub sites. True subglottic cancers are rare, accounting for only 5% of laryngeal cancers. The majority of tumour found in this area is a direct extension of glottic carcinoma rather than of true subglottic origin.

The differing embryological origins of each sub site can explain, in part, the variation in clinical behaviour of cancers from each site. The supraglottis, derived from the midline buccopharyngeal primordium and branchial arches 3 and 4, rich in lymphatics has a tendency for early bilateral regional lymphatic spread with approximately 50% of patients having nodal involvement at presentation. In contrast, the glottis, formed by the fusion of the lateral structures derived from the tracheobronchial primordium and arches 4 and 5 with a paucity of lymphatics, rarely spreads to regional lymphatics and tends to remain confined to the larynx (Lalwani, 2008). The Lymphatic drainage of all areas of the larynx and hence the sites of nodal metastasis is to levels II, III, IV and sometimes VI (see Figure 1.2). Fibroblastic membranes and ligaments further divide the larynx into the pre-epiglottic and para-epiglottic space and also acts as physical barriers, helping to prevent extra-laryngeal tumour spread (Lalwani, 2008).

### 1.1.3 The Oropharynx

The oropharynx is the area posterior to the oral cavity and extends from the plane of the superior surface of the soft palate above to the surface of the hyoid bone or floor of the valecullae below. It contains:

- Anterior sub sites (lymphatic drainage to level II nodes) – posterior one third of tongue and vallecula
- Lateral sub sites (lymphatic drainage to retropharyngeal nodes) – lateral wall, tonsil, tonsillar fossa and pillar
- Superior sub sites (lymphatic drainage to retropharyngeal and level II and III nodes) – inferior surface of soft palate, uvula

The oropharynx receives a sensory and motor nerve supply from the glossopharyngeal and vagal nerves. The hypoglossal nerve supplies motor innervation to the base of the tongue and the trigeminal nerve (V2, V3) supplies the motor and sensory innervation to the soft palate (Lalwani, 2008). Arterial blood supply is from branches of the external carotid artery. It is lined by smooth stratified squamous epithelium and has a rich lymphatic drainage system.

In the UK, the commonest site of oropharyngeal SCC is the lateral wall, incorporating the lateral sub sites above and accounting for 50% of tumours in this area. Between 65% and 77% of oropharyngeal tumours are complicated by nodal disease at presentation (with 20% of presentations having bilateral nodal disease) especially when the primary tumour is located in the posterior third of the tongue (Mukherji et al., 2001). The UK incidence in oropharyngeal SCC is rising; association with human papilloma virus (HPV) implicated.

### 1.1.4 The Nasopharynx

The nasopharynx is a cuboidal shaped muscular tube lined by pseudostratified columnar epithelium. It extends from the posterior choana anteriorly to the level of the free border of the soft palate along the plane of the airway. Lymphatic drainage is to the posterior cervical and jugulodiagastric nodes. Squamous cell carcinoma of the nasopharynx is rare in Western countries, with patients often presenting with late stage disease and associated lymphadenopathy as early disease is largely symptomless (Gleeson and Scott-Brown, 2008).

### 1.1.5 The Hypopharynx

This area extends from the plane of the superior border of the hyoid bone (or floor of the vallecula) to the plane corresponding to the lower border of the cricoid cartilage. The hypopharynx is lined by stratified squamous epithelium and has a rich lymphatic supply, draining to the deep cervical and paratracheal nodes (levels III-VI). It is sub-divided into the following areas:

- Postcricoid – from the level of the arytenoid cartilages to the inferior border of the cricoid cartilage
- Pyriform sinus – from the pharyngoepiglottic fold to the upper end of the oesophagus
- Posterior pharyngeal wall – from the superior border of hyoid to the inferior border of the cricoid cartilage and from the apex of one piriform fossa to the other

HNSCC in this region is relatively rare with only 10% of cases originating from this area (Stell et al., 2010). Presentation is often late with significant dysphagia and/or referred pain due to involvement of the glossopharyngeal and vagal nerves. The commonest sub site is the pyriform fossa with 65-85% of tumours found here (Lalwani, 2008). These tumours often extend medially through the aryepiglottic fold to invade the paraglottic space, anteriorly into the pre-epiglottic space, inferiorly to the post-cricoid region and often present with lymph node metastasis (67-75%; Lalwani, 2008). Post-cricoid tumours, although often extending inferiorly to the cervical oesophagus and laterally in the tracheo-oesophageal groove, are often less aggressive and present with nodal disease less often (20-30%; Lalwani, 2008), reflecting the differing lymphatic drainage between sites.

### 1.1.6 Histology of HNSCC

As described above, the majority of the UADT is lined by stratified squamous epithelium with malignant transformation resulting in squamous cell carcinoma (SCC) and accounting for 90% of tumours of the head and neck (Curado and Hashibe, 2009). Although other tumours such as salivary gland, thyroid, malignant melanoma and lymphoma can be found in this area, only SCC and its possible pre-malignant precursors will be discussed because of their prevalence.

#### *1.1.6.1 Leukoplakia*

Leukoplakia is a descriptive term relating to a 'white plaque' that cannot be characterised clinically or pathologically as any other disease. It is most likely found in the oral cavity, thought to arise from chronic irritation of the mucosa, e.g. pressure from an adjacent sharp tooth. Its pathological hallmarks include; hyperkeratosis (increased numbers of nucleated cells near the surface), acanthosis (elongation of rete pegs into the submucosa), abnormal orientation of cells, nuclear hyperchromatism, increased number of mitotic figures and an altered nuclear to cytoplasmic ratio. The frequency of dysplastic or malignant cell alterations within the lesion ranges from 16-39% (Neville and Day, 2002), however the consensus for the overall risk of malignant transformation is regarded as 3-6% (Dobrossy, 2005).

#### *1.1.6.2 Erythroplakia*

Erythroplakia is defined as a red mucosal plaque, usually found on the soft palate and tonsillar pillars. Although less common then leukoplakia, it has a higher incidence of dysplasia, which is noted on histological evaluation of most cases. The risk of malignant transformation is as much as seventeen times higher than in leukoplakia (Scully et al., 2003).

#### *1.1.6.3 Dysplasia*

Dysplasia is a histological diagnosis characterised by:

- Cellular atypia – increased nuclear to cytoplasmic ratio, cellular pleomorphism
- Loss of cellular maturity – increased mitotic figures, and
- Loss of stratification – abnormal progression from the basal to the more superficial layers

The World Health Organisation classified dysplasia into three groups (Pindborg et al., 1997) (Figure 1.4):

1) Mild – changes are limited to the lower third of the epithelial thickness.
2) Moderate – changes are limited to the lower two thirds of the epithelial thickness
3) Severe – in which the changes involve more than the lower two thirds of the epithelial thickness. Full thickness changes can also be described as carcinoma-in-situ with evidence of an intact basal membrane.

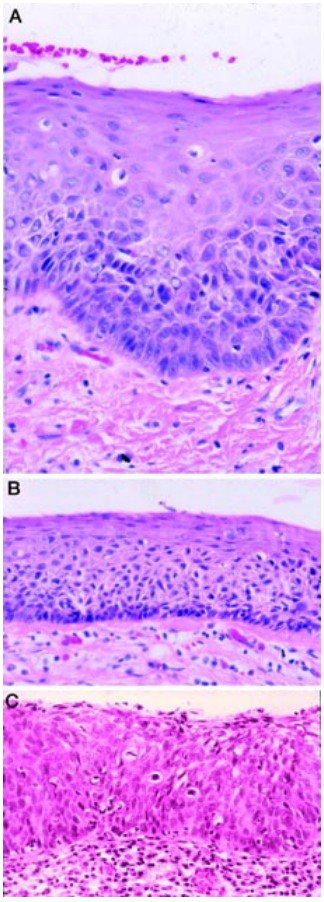



Figure 1.4 "Classic" or non-keratinizing dysplasia of the larynx. Stained with haemotoxylin and eosin, x 100 magnification. A, Mild dysplasia. B, Moderate dysplasia. C, Severe dysplasia with full-thickness replacement of the squamous epithelium by atypical, small, immature basaloid cells (Wenig, 2002).

### *1.1.6.4 Squamous Cell Carcinoma*

The progression of SCC from pre-cancerous lesions is summarised by a step-wise progression model (Figure 1.5). As with dysplasia, it is categorised into well, moderately and poorly differentiated lesions based on the degree of differentiation, cellular pleomorphism and mitotic activity. Invasion through the basement membrane (Figure 1.6) is the salient feature, distinguishing the development of frankly invasive disease from a carcinoma-in-situ.

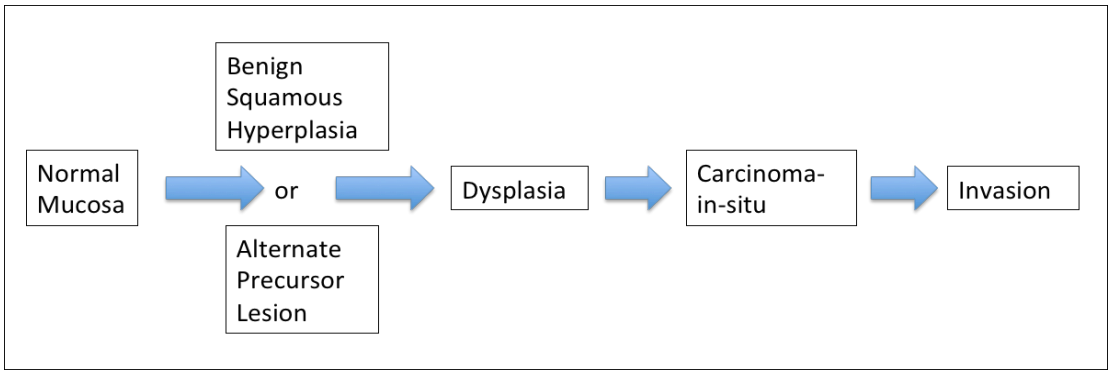



Figure 1.5 Diagram demonstrating the stepped progression model for HNSCC.

The clinical appearance of invasive SCC is quite variable and includes ulcerated, flat, exophytic, verrucoid, or papillary growths. Invasion can be as large cords or cohesive aggregates or can appear as single cells of small irregular aggregates. A desmoplastic stromal response (Figure 1.6), is a hallmark feature of invasive cancer, as is a foreign body reaction to keratin in the stroma (Wenig, 2002).

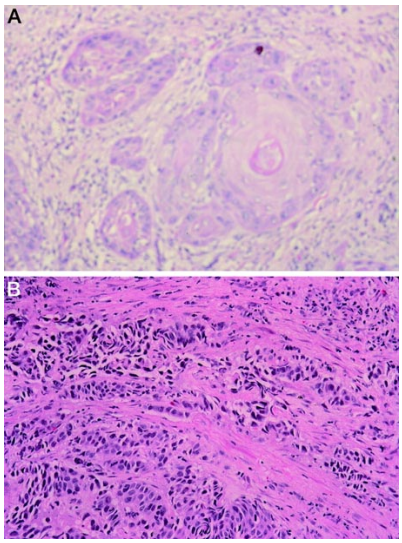



Figure 1.6 Image of invasive squamous cell carcinoma of the UADT. Stained with haemotoxylin and eosin, x 100 magnification. A, cohesive or nested growth pattern. B, dyscohesive growth pattern. In both illustrations, there is desmoplastic stromal response to the presence of invasive carcinoma (Wenig, 2002).

## 1.2 Epidemiology of HNSCC

The incidence of HNSCC varies significantly according to geographical location, sex and site, however, in general, the incidence of HNSCC has been in decline over the last decade in the Indian Subcontinent, East Asia, Western Europe and the United States in men and levels have generally stabilised in women (Parkin et al., 2005). North and Eastern Europe has seen an increase in oral cavity, tongue and pharyngeal cancer in both men and women (Curado and Hashibe, 2009).

Oral cavity SCC is the most common form of HNSCC worldwide, accounting for 274,000 cases in 2002, with almost two-thirds of cases in men (Parkin et al., 2005). Areas of high incidence include Melanesia (31.5 per 100,000 in men and 20.2 per 100,000 in women), Western Europe (11.3 per 100,000 in men), and south Asia (12.7 per 100,000 in men) with patterns of incidence reflecting risk factors, such as tobacco/alcohol use in Western Europe, and the chewing of betel nut in South-central Asia and Melanesia.

There were 159,000 new cases and 90,000 deaths due to laryngeal carcinoma worldwide in 2002 (Parkin et al., 2005). It is less common in women (M:F sex ratio, 7:1). Areas of high incidence include Southern Europe (France, Italy, Spain), Eastern Europe (Russia, Ukraine), South America (Uruguay, Argentina) and Western Asia (Turkey, Iraq) where it accounts for 4.7% of cancers in men compared with 2.4% worldwide (Parkin et al., 2005).

Globally, oropharyngeal cancer accounted for 174,200 cases in 2002, again almost two thirds occurring in men (Parkin et al., 2005). Nasopharyngeal cancer accounts for very few cases of HNSCC worldwide with incidence rate less than 1 in 100000 for the most part. It is however common in some ethnic groups (as further explained in section 1.3.5) with the highest rate found among the Chinese, particularly in Hong Kong and Singapore (Goldsmith et al., 2002). Males are more often affected than females (M:F sex ratio, 2.3:1), and in most populations risk increases with age (Parkin et al., 2005).

In the UK, HNSCC represents 3% of all diagnosed cancers. The 2007 incidence rates are summarised in Table 1.1. Patients with oral cancer are increasing

disproportionately in comparison with other sites of HNSCC (Figure 1.7). This is thought to be partly due to previous increased use of tobacco and alcohol within the population as this tumour is highly associated with smoking and drinking. The increased incidence of oropharyngeal SCC is also likely to be responsible as oropharyngeal SCC is often wrongly included within the oral cavity group when cases are reported.

| *Cancer Site* | *Male* | *Female* | *Total (%)* |
|---|---|---|---|
| Oral Cavity (total) | 2,782 | 1,544 | 4,326 (55) |
| Lip | 191 | 81 | 272 (3) |
| Mouth | 1,000 | 695 | 1,695 (22) |
| Tongue | 1,061 | 581 | 1,642 (21) |
| Larynx | 1,844 | 361 | 2,205 (28) |
| Oropharynx | 812 | 272 | 1,084 (14) |
| Nasopharynx | 167 | 74 | 241 (3) |

Table 1.1 The number of new cases of HNSCC in the UK in 2007. Adapted from figures by Cancer Research UK (http://info.cancerresearchuk.org/cancerstats/)

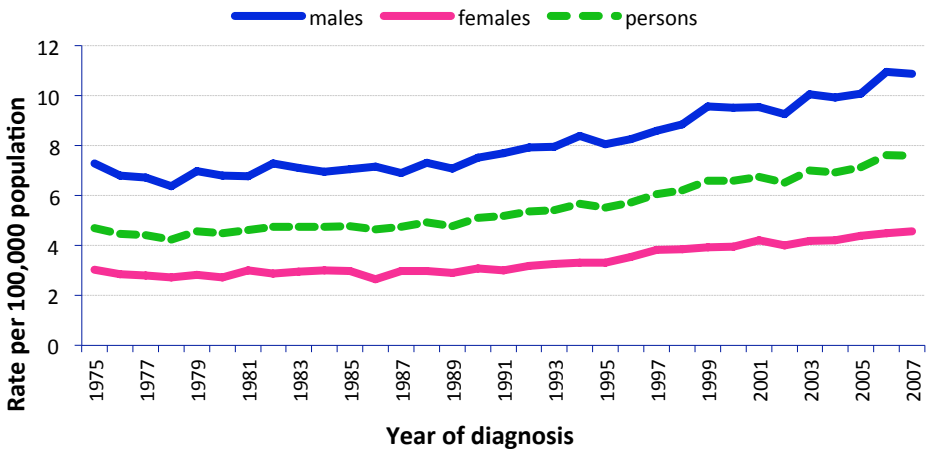



Figure 1.7 Graph of age-standardised incidence rates, by sex, oral cancer, UK, 1975-2007. Adapted from figures by Cancer Research UK (http://info.cancerresearchuk.org/cancerstats/)

Regional variations in incidence are seen throughout the UK and statistics for laryngeal cancer, demonstrating highest rates in Scotland, are given below (Figure 1.8). One reason for this is variations in socio-economic status, Thorne et al. (1997) found that the incidence of oral cancer in men was significantly higher in the most economically deprived group.

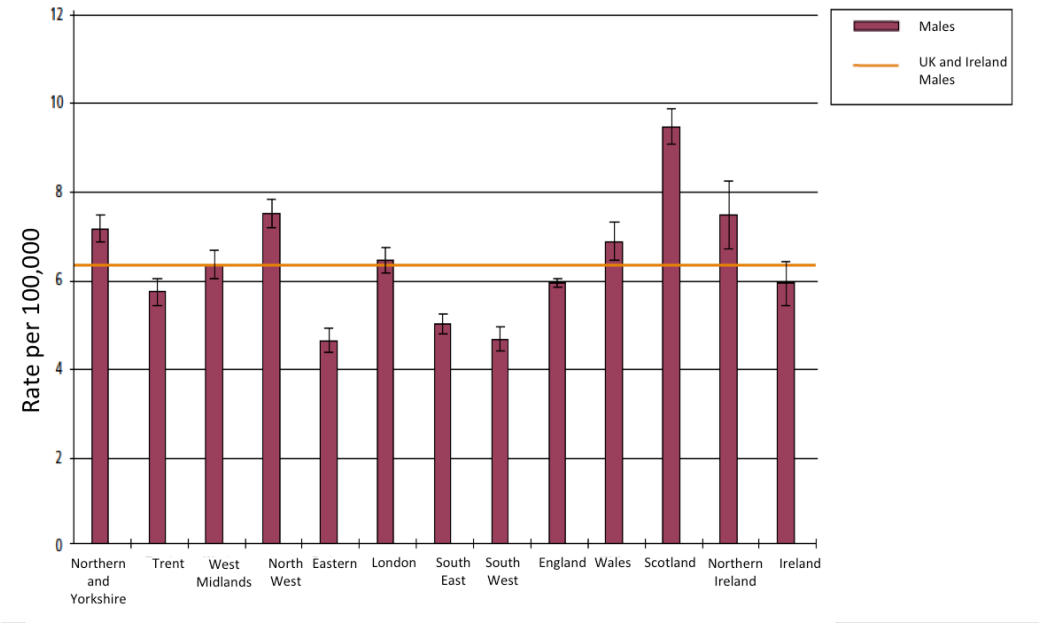



Figure 1.8 Graph showing the incidence of laryngeal UK by country and region, 1991-1999 (Cancer Atlas of the UK and Ireland, 1991-2001) http://www.statistics.gov.uk).

## 1.3 Aetiology of HNSCC

### 1.3.1 Tobacco

Tobacco use is known to be the main causative factor in laryngeal cancer, with 98% of patients being smokers (Tuyns et al., 1988). Likewise, oral cavity and oropharyngeal cancer are most strongly associated with tobacco and alcohol consumption (although the recently described association of oropharyngeal SCC with HPV may be more important in some patients). In both of these cancers, higher tobacco consumption and increased length of exposure confer an increased risk of malignancy with the added effects of alcohol seemingly synergistic (Kabat et al., 1994). Epidemiological studies citing figures for relative risks (RR) are summarised in Table 1.2:

| *Author, year of publication* | *SCC sub site* | *Findings* |
|---|---|---|
| Wynder et al. (1976) | Laryngeal | RR 4.4 <10 cigs/day |
| | | RR 34.4 >40 cigs/day |
| Falk et al. (1989) | Laryngeal | RR 4.4 >20 cigs/day |
| | | RR 10.4 >40 cigs/day |
| Merletti et al. (1989) | Oral/oropharyngeal | RR 4-6 medium to high consumption of tobacco |
| Maier et al. (1992) | UADT | RR 23.4 >60 pack years |
| Macfarlane et al. (1996) | UADT | RR 2.4 7 cigs/day |
| | | RR16.4 >25 cigs/day |
| Gandini et al. (2008) | Laryngeal | RR 6.98 |
| (meta-analysis) | Pharyngeal | RR 6.76 |
| | UADT | RR 3.57 |
| | Oral | RR 3.43 |

Table 1.2 Summary of publications demonstrating relative risk of laryngeal cancer attributed to tobacco consumption.

The method of tobacco use often reflects incidence of specific sub sites of HNSCC; areas where chewing tobacco is prevalent (e.g. India) have high rates of oral

cavity and hypopharyngeal cancer (Sapkota et al., 2007), whereas where reverse smoking is common-place, palatal SCC predominates (Squier, 1984).

Tobacco is known to contain a variety of carcinogenic compounds, with over 60 compounds identified (Kuper et al., 2002). Potent carcinogens include polycyclic hydrocarbons such as benzo-pyrene, nitrosamines and aromatic amines (Zaridze and Peto, 1986).

### 1.3.2 Alcohol

Alcohol is thought to potentiate tobacco related carcinogenesis rather than act as a carcinogen *per se*. The mechanisms for this are not fully understood. It was thought that ethanol may enhance the contact of the tobacco carcinogens to the mucosal lining of the upper aero digestive tract (Blot et al., 1988). This does not however fully explain the persistence of elevated risk after cessation of drinking. Other studies have suggested a direct chromosomal effect with altered alkylated DNA repair (Garro et al., 1986, Mufti et al., 1988). More recently, several groups have suggested that the acetaldehyde component is a major factor, causing DNA damage by the formation of DNA adducts (Brooks and Theruvathu, 2005, Boffetta and Hashibe, 2006).

The type of alcohol is also important; a Danish population study (Gronbaek et al., 1998) of 28,180 found that people who only drank beers and/or spirits had a higher RR of developing HNSCC in comparison to those who included wine in their alcohol intake. More recently, pooled data from 15 case controlled studies on HNSCC (Purdue et al., 2009) including 9,107 cases and 14,219 controls found a similar RR in liquor-only and beer-only drinkers for ethanol-standardised consumption frequency of >30 drinks per week. Among wine-only drinkers, the odds ratios for moderate levels of consumption frequency approached the null, whereas those for higher consumption levels were comparable to those of drinkers of other beverage types. Thus, whilst there is a strong association between beer and spirit consumption and HNSCC, those drinking a similar quantity of wine are at a lower risk, with moderate consumption possibly offering a degree of protection.

### 1.3.3 Human Papilloma Virus (HPV)

Known to be an important risk factor in SCC of the cervix, HPV has now been implicated in HNSCC. DNA from HPV subtypes 16 and 18 is frequently detected in HNSCC. A recent meta-analysis of 62 studies examining paraffin-embedded biopsies of HNSCC found the pooled prevalence of HPV DNA by either PCR-detection or *in-situ* hybridisation to be 34.5% (excluding oral), with a higher rate of 38.1% in oral SCC (Termine et al., 2008).

The molecular and histopathological characteristics of HPV associated HNSCC are not yet fully understood, but current evidence suggests that tumours with detectable HPV express E6/E7 mRNA, which is known to inactivate the tumour suppressor gene products p53 and pRb (Retinoblastoma protein) products, potentiating cell immortalisation (Rampias et al., 2009).

Although numerous studies have detected the presence of HPV, controversy still remains as to whether it is an incidental finding or plays an important role in the initiation and promotion of SCC. Emerging evidence, particularly regarding the association of HPV with oropharyngeal SCC suggests that HPV positive patients have a better outcome than HPV-negative ones despite presenting at a more advanced stage (Schlecht, 2005, Ang et al., 2010). In a recent study by Ang et al. (2010) the 63.8% of patients with HPV-positive oropharyngeal cancer (206 of 323) had improved 3-year rates of overall survival (82.4% vs. 57.1%) among patients with HPV-negative tumours. In addition, after adjustment for age, race, tumour and nodal stage, tobacco exposure, and treatment assignment, HPV-positive patients had a 58% reduction in the risk of death, leading the authors to conclude that tumour HPV status is a strong and independent prognostic factor for survival among patients with oropharyngeal cancer.

The evidence of increased incidence of HPV in oropharyngeal SCC, reduced age of incidence and altered prognosis, all support the opinion that tumourigenesis in HPV positive oropharyngeal positive SCC may be due to an alternative, though overlapping, mechanism separate to the 'classic' HNSCC carcinogenesis *per se* (Li and Sturgis, 2006).

### 1.3.4 Epstein-Barr Virus (EBV)

There is strong evidence for the role of EBV in the development of nasopharyngeal carcinoma (NPC), however the pandemic nature of this infection and the distinct geographical variations of NPC worldwide mean that this is not the only causative factor. The virus, not normally found in epithelial cells of the nasopharynx, is often present in NPC tumour cells as well as dysplastic and carcinoma *in situ* lesions (Pathmanathan et al., 1995). The virus alone cannot drive normal cells towards carcinoma development; it is thought that loss of heterozygosity, possibly as a result of inherited traits (Chinese ethnicity) (Goldsmith et al., 2002), exposure to dietary factors (salted fish) (Morton and Benjamin, 1989) and other environmental cofactors, is an early occurrence in NPC. It is within these low-grade, pre-invasive lesions, subsequent to further genetic and epigenetic alterations, where EBV infection occurs. The expression of EBV latent genes (encoding latent membrane proteins LMP1, LMP2A and LMP2B) provides growth and survival advantages to these infected cells, ultimately leading to the development of NPC (Shah and Young, 2009). The role of EBV in tumour formation, although unique to NPC, clearly demonstrates the role of viral aetiology in cancer

### 1.3.5 Genetic Alterations in HNSCC

The development of HNSCC is a multistep carcinogenesis process involving the progressive loss of homeostatic control of cell growth and death (Califano et al., 1996). Alterations in the expression of cell cycle regulatory proteins involved in DNA repair, proliferation, apoptosis, invasion and angiogenesis result from the activation of proto-oncogenes and the inactivation of tumour suppression genes. This ultimately leads to an unbalanced mitogenic signal and consequent aberrant cell proliferation. Key events in this process include inactivation of the p53 tumour suppressor gene, inactivation of the cyclin-dependent kinase (CDK) inhibitor p16 and over-expression of epidermal growth factor receptor (EGFR)(Hardisson, 2003).

#### *1.3.5.1 p53*

The p53 gene encodes a tumour suppressor protein with the ability to activate DNA repair proteins after damage, induce growth arrest by holding the cell

cycle at the G1/S regulation point on DNA damage recognition and initiate apoptosis if the DNA damage proves to be irreparable (Hollstein et al., 1991). A diagrammatic representation of the activation/inactivation of p53 is shown in Figure 1.9. Studies have shown a presence of the mutated form in 25-80% of cases of HNSCC (Fan, 2001). Mutations are more common in patients with a history of alcohol and tobacco use; the type, frequency and location of p53 mutations have been associated with specific carcinogenic agents. Base transitions, typically G →T substitutions and transversions, for example are thought to be related to exposure to exogenous exposures such as tobacco smoke (Pfeifer et al., 2002) and are more likely to be found in HNSCC compared to other sites, e.g. colon (Olshan et al., 1997).

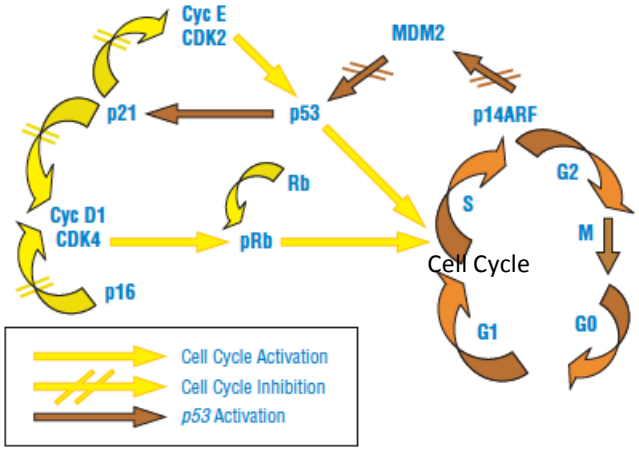



Figure 1.9 Diagram demonstrating cell cycle regulation by Rb and p53 tumour suppressor proteins. Adapted from (Yip et al., 2006). Inactivation of Rb and p53 proteins occurs by phosphorylation, enabling the cell cycle to progress from the G1 to the S phase. Kinase function of CDK4 is activated by cyclin D1 and inactivated by p16 proteins. Cyclin E and p21 control the activation and inactivation of CDK2, respectively. Ubiquitination of p53 takes place by complexing with MDM2 that is blocked by p14ARF.

The presence of a p53 mutation is likely to be of prognostic significance. Poeta et al. (2007) correlated the presence of any p53 mutation, as found in 224 of their 440 HNSCC tumours, with decreased overall survival. In addition, a stronger association was seen with disruptive mutations leading the authors to conclude that the site of the p53 mutation is crucial, with mutations in the DNA-binding domain of

most prognostic importance. In agreement, p53 mutations in direct DNA contact areas resulted in accelerated tumour progression and reduced therapeutic responsiveness in HNSCC (Erber et al., 1998, Temam et al., 2000).

### *1.3.5.2 Cyclins*

Cyclins, part of the cell cycle regulation process, are often altered in malignancies. Cyclin D1 activates cyclin-dependent kinase 4 (CDK4), allowing the cell to proceed through a checkpoint in the cell cycle, whereas p16 inhibits CDK4, acting as a tumour suppressor as shown in Figure 1.10. In a population-based case-control study of 698 cases and 777 controls, immunohistochemical staining of variant cyclin D1 protein overexpression resulted in an increased protein half-life and was associated with an increased risk of HNSCC in addition to poorer survival outcomes (Marsit et al., 2008). Furthermore, amplification of cyclin D1 or deletion of p16 in tumours was associated with recurrence, distant metastasis, and reduced survival at 36 months in a study of 103 HNSCC tumours by Namazie et al. (2002). The finding of alterations in cyclin D1 and p16 in biopsies of mild and moderate dysplasia is evidence for their involvement in the early stages of carcinogenesis (Papadimitrakopoulou et al., 2001).

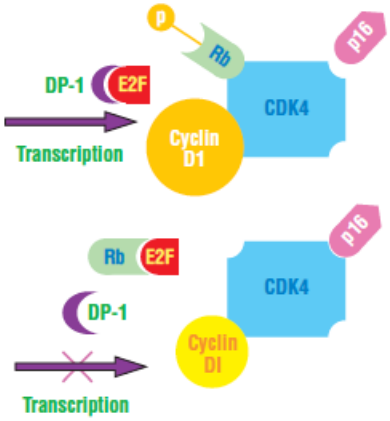



Figure 1.10 Diagram demonstrating the activation and inhibition mechanisms of CDK4. Activation and inactivation of p16 by deletion, methylation, or mutation and/or by amplified expression of cyclin D1 leads to increased phosphorylation of Rb. However, increased expression of p16 and reduced expression of cyclin D1 results in hypophosphorylated Rb binding to E2F transcription factor leading to the inactivation of transcription. Adapted from Yip et al., 2006.

#### *1.3.5.3 EGFR*

EGFR (or erbB1) is a membrane receptor and a member of the tyrosine kinase family (HER RTKs). Binding of the ligands (EGF and transforming growth factor-β, TGFβ) to the receptor's extracellular domain induces EGFR dimerization, resulting in the activation of receptor tyrosine kinase activity and EGFR autophosphorylation on specific tyrosine residues (Schlessinger, 2000). Various intracellular signalling pathways such as Ras/Raf/MEK/MAPS that control cellular proliferation, migration and differentiation can then be initiated (Schlessinger, 2000, Hubbard and Miller, 2007).

Alterations in the EGFR signalling pathway can cause malignant transformation through various mechanisms, including receptor over-expression, alteration in dimerization and deficiency of specific phosphatases (Grunwald and Hidalgo, 2003). The over-expression of EGFR has been reported in 73–92% of HNSCC (Hiraishi et al., 2006, Laimer et al., 2007, Sarkis et al., 2010). EGFR over-expression has been found to correlate with more aggressive disease, resistance to both chemotherapy and radiotherapy and poor prognosis (Ang et al., 2002, Kong et al., 2006).

### 1.3.6 Summary

After considering the various aetiological factors and the genetic pathways implicated in the development of HNSCC, it is obvious that numerous factors are involved. Also highlighted, is the difference in protein expression, not just between tumour sub sites but also within tumours of the same location. An appreciation of this, combined with other patient related factors such as the immune response and co-existing morbidities, perhaps goes someway into explaining, or more importantly appreciating, the heterogeneity of tumour response to standard treatment.

## 1.4 Staging of HNSCC

The staging of HNSCC is a system designed to convey the disease extent or relative severity. It can give an indication of prognosis, aid the clinician in planning treatment, assist in evaluation of treatment results and facilitate exchange of information between centres. Staging is determined by clinical and histopathological means, dependent on extent of primary tumour (T), absence or presence and extent of regional lymph node metastases (N) and absence or presence of distant metastases (M). This system was first described by Pierre Denoix in 1944 (Denoix, 1952) and later modified by the World Health Organisation (WHO) and the Union for International Cancer Control (UICC). The American Joint Committee on Cancer (AJCC) also developed a staging system, however, the TNM system has now been adopted by all, with both the current (sixth) edition of the AJCC cancer manual and the current sixth edition of the UICC (Sobin et al., 2002), both published in 2002, containing identical classifications. The TNM system is further adapted for each tumour sub site of the head and neck (see Appendix 1) but can be generalised for all tumour types as shown below (Sobin et al., 2002).

| *T/N/M* | *Definition* |
|---|---|
| TX | Primary tumour cannot be assessed |
| T0 | No evidence of primary tumour |
| Tis | Carcinoma in situ |
| T1,T2,T3,T4 | Increasing size and /or local extent of tumour |
| NX | Regional lymph nodes cannot be assessed |
| N0 | No evidence of regional lymph node metastasis |
| N1,N2,N3 | Increasing involvement of regional lymph nodes |
| MX | Distant metastasis cannot be assessed |
| M0 | No distant metastasis |
| M1 | Distant metastasis |

Table 1.3 Summary of TNM staging (Sobin et al., 2002).

The status of regional lymph node metastasis in HNSCC is of great importance and irrespective of sub site, is as follows:

| *N* | *Definition* |
|---|---|
| N1 | Metastasis in a single ipsilateral node, ≥3cm |
| N2a | Metastasis in a single ipsilateral lymph node >3cm but ≤6cm |
| N2b | Metastasis in multiple ipsilateral lymph nodes ≤6cm |
| N2c | Metastasis in bilateral or contra lateral lymph nodes ≤6cm |
| N3 | Metastasis in a lymph node >6cm |

Table 1.4 Classification of nodal metastasis in HNSCC (Sobin et al., 2002)

Although the TNM is a global, standardised, system applicable to all HNSCC, there are limitations. Only the anatomical extent of the tumour is taken into account; other factors known to influence outcome and disease progression are not taken into account (e.g. extra-capsular spread of nodal disease). Other staging systems are more specific e.g. the tumour and node interger score system, TANIS (Carinci et al., 1999) and the Halls system (Lydiatt et al., 2001), however are more time consuming and therefore less user friendly when applied to the clinical setting.

The TNM stages are further grouped into stages (Sobin et al., 2002), reflecting survival rates.

| *Stage* | *T* | *N* | *M* |
|---|---|---|---|
| 0 | Tis | N0 | M0 |
| I | T1 | N0 | M0 |
| II | T2 | N0 | M0 |
| III | T1,T2 | N1 | M0 |
| | T3 | N0,N1 | M0 |
| IVa | T1,T2,T3 | N2 | M0 |
| | T4a | N0,N1,N2 | M0 |
| IVb | Any T | N3 | M0 |
| | T4b | Any N | M0 |
| IVc | Any T | Any N | M1 |

Table 1.5 HNSCC Stages I-IV based on the TNM Classification (Sobin et al., 2002)

## 1.5 Management of HNSCC

When considering the primary tumour, early stage squamous carcinoma (T1 and T2) at most sites is treated by a single modality, either surgery, or radiotherapy (see figure 1.11). More advanced tumours (T3 and T4) are commonly managed by a combination of surgery and postoperative radiotherapy or chemo-radiotherapy with or without induction chemotherapy (Choong and Vokes, 2008).

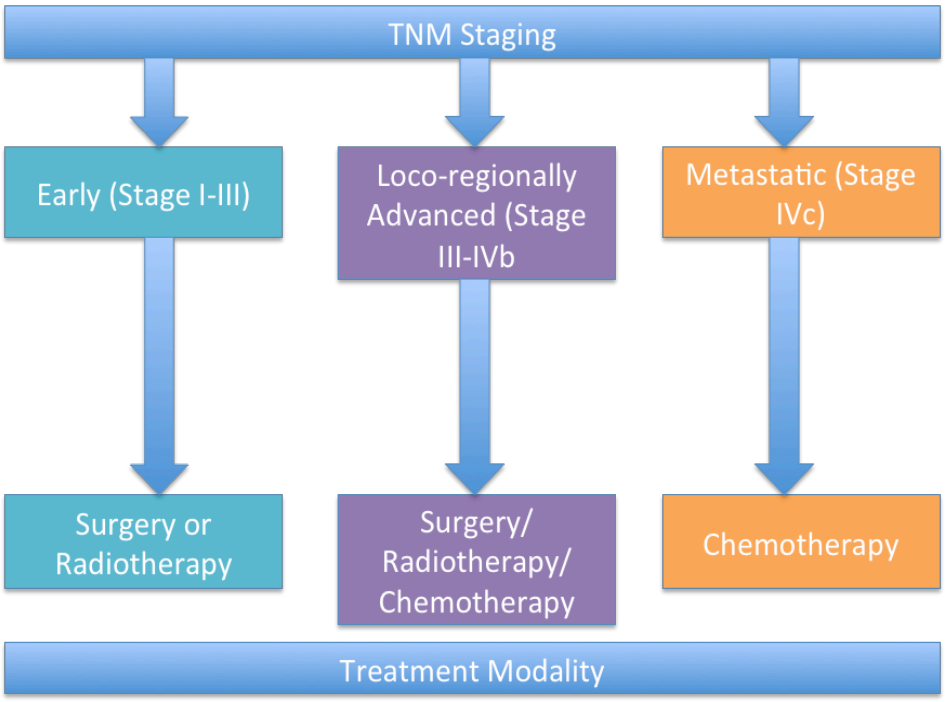



Figure 1.11 General schema for the management of HNSCC, adapted from (Choong and Vokes, 2008).

The current 'standard' treatments based on tumour site and stage are summarised below

### 1.5.1 Management of Cervical Metastases

Elective treatment of the N0 neck depends on the site and histology of the primary tumour and in general consists of a selective neck dissection or radiotherapy in those tumours with an incidence of micro-metastases of more than 20% (Weiss et al., 1994). In the presence of cervical metastases, neck dissection is recommended. The exception however is in small volume nodal disease (<2 cm), when it can be successfully encompassed in a radiation field. For example in N1 necks in tonsillar

carcinoma, surgical and radiotherapy results are comparable (Robson, 2001). In some advanced cases, metastases may be considered unresectable for technical reasons, e.g. infiltration of prevertebral musculature, brachial plexus etc. In some patients, although it may be possible to resect the nodal disease, it is considered to be too functionally disabling, or the patient co-morbidity considered too risky to attempt to do so. A patient may choose not to undergo surgical treatment. In such circumstances radiotherapy or, in fit patients, chemo-radiotherapy, may then be offered as primary treatment.

The type of neck dissection performed is classified in accordance with the consensus statement on the classification and terminology of neck dissection (Robbins et al., 2008). A radical neck dissection describes the removal of lymph node levels I-V, the sternocleidomastoid muscle, internal jugular vein and accessory nerve. This technique, originally described by Crile in 1906 (Rinaldo et al., 2008) is the gold standard of neck dissection, but due to its inevitable morbidity is now reserved for advanced nodal disease. If possible, a modified neck dissection is now performed, with the aim of preserving one or more of the non-lymphatic structures above.

An appreciation of the patterns of nodal metastatic spread from individual tumour sites within the head and neck, based on the clinical studies of Lindberg (1972), has led to the increased use of the selective neck dissection, allowing for the preservation one or more lymph node groups thus limiting morbidity. An extended neck dissection describes the additional removal of other lymph node groups (parapharyngeal, paratracheal, superior mediastinal), or other non-lymphatic structures (carotid artery, vagus nerve, hypoglossal nerve, prevertebral muscle).

In addition to surgery, post-operative radiotherapy is given when there are more than two nodes pathologically involved, when the nodal disease is found in more than one surgical level, if there are any nodes more than 3cm in size and / or if there is extra-capsular rupture (Gleeson and Scott-Brown, 2008). The timing of post-operative radiotherapy is of importance, within 6 weeks of surgery being optimal as delay of >6 weeks decreases the likelihood of cure (Vikram, 1979, Schiff et al., 1990).

### 1.5.2 Laryngeal Carcinoma

Treatment selection varies based on precise anatomical location with glottic and supraglottic tumours differing. In general radiotherapy and conservation surgery alone are options for T1-2, N0 lesions whereas combined surgery and radiotherapy is used for advanced (high volume T3 and T4) and nodal disease.

Regarding glottic tumours, for T1-2, N0, single modality treatment is recommended; traditionally radical radiotherapy, however recent interest in endoscopic laser techniques has led to their increased use. A Cochrane review published in 2002 (Dey et al., 2002), comparing radiotherapy, open surgery and endolaryngeal excision (with or without laser) for early (T1, T2) glottic tumours suggests they confer similar survival advantages. This was echoed by a meta-analysis by Higgins et al. (2009) comparing the survival outcomes for laser resection and radiotherapy for early stage glottic cancer; although it was suggested there was an improved voice quality following radiotherapy.

In stage 3 disease (T3 or T1-3, N1), tumours can vary in their bulk, but most are managed with the aim of laryngeal preservation. The current trend is towards treatment with neoadjuvant and/or concomitant chemotherapy with radiotherapy, with surgery reserved as salvage for residual disease as assessed approximately 3-4 months after treatment. In certain limited tumours partial open surgery or laser resection can be attempted.

In T4 tumours, surgery (usually total laryngectomy) with selective (in the N0 neck) or modified radical (in the N+ neck) neck dissection with or without post-operative radiotherapy is the treatment of choice. To date, there are no randomised controlled trials comparing different surgical treatments for advanced laryngeal cancer, but the Veterans Administration trial (The Department of Veterans Affairs Cooperative Studies Program, 1991) (see section 1.7) recommends total laryngectomy and voice rehabilitation in T4 tumours and is unequivocal towards radiotherapy or surgery for T3 tumours. In tumours of relatively low bulk and limited invasion of laryngeal framework and low bulk lymph nodes, neoadjuvant and/or

concomitant chemotherapy with radiotherapy is an option, however salvage laryngectomy is likely in approximately 50% of all cases.

Supraglottic tumours have a high incidence of overt and occult nodal metastatic disease and this is reflected in their treatment. As with glottic tumours, early stage tumours are usually treated with a single modality and advanced tumours with combined surgery and radiotherapy. In T1-2, N0 stage tumours, conservation surgery or radiotherapy can achieve similar cure rates (Spriano et al., 1997), the choice depending on the tumour distribution and the patient's co-morbidities. For T3-4, N0-3 tumours the treatment of choice is surgery, i.e. subtotal or total laryngectomy with neck dissection plus radiotherapy if indicated.

For the less common subglottic laryngeal tumours, radiotherapy is indicated in early (T1, T2 stages) whereas T3 and T4 tumours are generally managed by laryngectomy.

For early stage laryngeal tumours, the survival benefits associated with different treatment modalities is negligible, for advanced staged tumours however, much debate still exists and of current interest is the role of chemotherapy in its adjuvant, neoadjuvant and synchronous settings as will be discussed further in section 1.7.

### 1.5.3 Oropharyngeal Carcinoma

Surgery and radiotherapy give comparable results in T1 and T2, N0 disease; data from the Scottish Head & Neck Cancer audit (1999–2001) demonstrated no significant difference in disease-specific survival between early stage disease treated with primary surgery (42 patients) or non-surgical treatment (30 patients). The choice therefore depends on the site of the lesion and the patient's medical condition and preferences and based on primarily the above evidence, a recent consensus statement (Robson and Paleri, 2010) recommended that early stage disease can be treated by surgical or non-surgical methods.

For stage 3 disease (T1/2 N1, T3N0) there are various treatment options:

1) Neoadjuvant chemotherapy with cisplatin and 5-fluorouracil (5FU), particularly if there is bulky nodal disease or TPF (Taxotere, Cisplatin and 5FU) for medically fit, unresectable T4 N3 patients followed by radical radiotherapy with concurrent cisplatin.

2) Surgery and post-operative radiotherapy.

3) Surgery and post-operative chemoradiotherapy if high risk i.e. positive margins or extra nodal spread.

4) Radiotherapy +/- chemotherapy with brachytherapy boost for base of tongue cancers.

Stage 4 tumours tend to be inoperable or have functionally poor operative results. Patients may be treated with aggressive chemoradiotherapy, a split course or short course radiotherapy or palliative chemotherapy.

There is inadequate evidence to support either surgical or non-surgical treatment for advanced disease (Robson and Paleri, 2010) and to date no randomised data are available comparing surgery and radiation +/− chemotherapy in oropharyngeal cancer. The role of surgery versus chemoradiotherapy remains controversial, with some groups finding that patients treated with non-surgical modalities have better functional outcomes (Parsons et al., 2002) and others (Kim et al., 2010) finding better quality of life outcomes in those managed with surgery.

As described in section 1.3.4, recent evidence from Ang et al. (2010), concurring with evidence from a previous meta-analysis (Ragin and Taioli, 2007), has demonstrated a better prognosis for HPV positive than negative tumours in oropharyngeal cancer. There is emerging evidence that patients with HPV positive oropharyngeal cancer should be offered less aggressive treatment than HPV negative cancers because of the more favourable prognosis (Robson and Paleri, 2010), however these findings require further validation.

### 1.5.4 Nasopharyngeal Carcinoma

NPC is extremely radiosensitive and the mainstay of treatment for primary local and regional disease is invariably radiotherapy, almost irrespective of the stage

of the disease (Gleeson and Scott-Brown, 2008). For patients with advanced disease, chemotherapy appears to enhance the overall treatment results. With the addition of concurrent (cisplatin) based chemoradiation, a significant short-term progression-free survival benefit, up to a mean disease free survival of three years was shown by two independent phase III studies (Al-Sarraf et al., 1998, Chan et al., 2002) and by meta-analysis (Huncharek and Kupelnick, 2002).

### 1.5.5 Hypopharyngeal Carcinoma

Hypopharyngeal cancer has a poor prognosis due to its relatively late presentation. Local control is best achieved in advanced and node positive disease by combined surgery and radiotherapy. Conservative surgery (including larynx preservation) followed by radiotherapy or the option of radiotherapy with neoadjuvant and/or concomitant chemotherapy is commonly used for earlier stage disease. Evidence suggests that there is little difference between radiotherapy and surgery for T1-3 pyriform fossa tumours (Jones et al., 1994) and node negative post-cricoid tumours (Dische et al., 1997). Results from the European Organisation for Research and Treatment of Cancer (EORTC) (Lefebvre et al., 1996) suggest that laryngeal preservation is possible in advanced pyriform fossa disease by using induction cisplatin and 5FU and adapting treatment, i.e. further chemotherapy and radical radiation or surgery following irradiation depending on initial chemotherapy response. There is also evidence for the addition of adjuvant chemotherapy to existing surgical and radiotherapy regimens in some circumstances (see section 1.7)

If conservative surgery is not possible then a laryngectomy and partial pharyngectomy (+/- flap) or total pharyngo-laryngectomy (plus stomach transposition or jejunal graft transfer) and neck dissection is necessary depending on the exact site. In addition, a selective neck dissection (levels II, III, IV +/- VI) is performed in the N0 and sometimes the N1 neck. Radical or modified radical dissection is performed for N2-3. Bilateral dissection is required for mid-line tumours due to the frequency of bilateral neck node metastasis.

### 1.5.6 Summary

The management of HNSCC varies for each sub site and whilst in some cases optimal regimens are potentially understood (e.g. nasopharyngeal) the role of chemotherapy in other tumours is still questioned. Using the incorrect treatment modality may be of serious consequence to the patient, either in terms of under- or overtreatment leading to death or an intolerable quality of life. This highlights the need for tools for predicting treatment response.

## 1.6 Radiotherapy

Radiotherapy induces cell death largely by damage to nuclear DNA, with breakage leading to irreversible loss of the reproductive integrity of the cell and eventual cell death (Rosen et al., 2000). Damage can be caused directly by the radiotherapy beam ionizing the atoms which make up the DNA chain, or indirectly, as a result of the ionization of water, forming free radicals, notably hydroxyl radicals, which then damage the DNA. The therapeutic mechanism for radiation is based on the intrinsic ability of normal cells to repair damage, whilst tumour cells, particularly radiosensitive ones, possess inappropriate mechanisms of damage detection and repair leading to the activation of apoptotic pathways and ultimately programmed cell death (Rosen et al., 2000). Radiotherapy is indicated as a primary treatment as described above and as a post-operative addition in the case of positive resection margins, extracapsular lymph node spread, two or more histological positive nodes or multiple nodes at multiple levels, in most tumour sub-sites (Gleeson and Scott-Brown, 2008).

Conventional radical, fractionated, treatment dosage is usually 1.8-2 Gy per day, administered 5 days a week for 5-7 weeks to a total of 66 to 70 Gy, depending on the particular clinical situation (Gleeson and Scott-Brown, 2008). The exception is small volume (e.g. T1 glottic tumours) disease when 55Gy in 20 fractions over 4 weeks is given. For adjuvant (post-operative) radiotherapy, 60Gy in 30 daily fractions (or if high risk / macroscopic residual 66Gy in 33 fractions) is given.

Efforts to improve the loco-regional control by altering the fractionation have had some successful. Hyperfractionation (Fu et al., 2000, Horiot et al., 1992) (e.g. multiple smaller fragments of 1.2Gy twice a day over the same time period) and accelerated hyperfractionation (Fu et al., 2000, Overgaard et al., 2003) (delivery of the total dose over a shorter time period) have been compared with conventional fractionation. Altered regimens have led to a 10 to 20% improvement in local control rates, but with less clear effects on survival and increased acute toxicity (Fu et al., 2000).

More recently, Intensity Modulated Radio Therapy (IMRT) is being used for a number of head and neck sites. Multiple beams of varying intensities are used to create irregular shapes, if necessary, with concave contours. This technique allows for the more precise delivery of radiation and optimization of the dose intensity to specific volumes, while sparing the dose to critical normal structures such as the parotid glands (Caglar and Allen, 2007) . IMRT has been shown in a number of clinical sites to improve local control and decrease side effects (Dawson et al., 2000, Kwong et al., 2004) and improve quality of life when compared to conventional radiotherapy (Graff et al., 2007). Most recently, principally from a UK randomised trial of 88 patients, IMRT reduced radiation induced xerostomia (the main long term side effect of standard radiotherapy) from 75% to 39% at 12 months after treatment (Nutting et al., 2009).

## 1.7 Chemotherapy

In HNSCC, chemotherapy is employed either as a palliative therapy for patients with metastatic disease or administered simultaneously/ prior to radiotherapy in order to induce radio-sensitization of the tumour. The combination of chemotherapy and radiotherapy in head and neck cancer has been the subject of many studies in recent years, the rationale founded upon high response rates of HNSCC to a variety of chemotherapy agents including methotrexate, vinblastine, bleomycin, 5FU, cisplatin, carboplatin and the taxanes, either alone or in combination (Stupp et al., 1994).

Chemotherapy may be administered before definitive treatment (induction or neo-adjuvant), simultaneously with radiotherapy (concomitant or concurrent), or after surgery (adjuvant) in loco-regionally advanced (i.e. T3/T4) HNSCC (Figure 1.12).

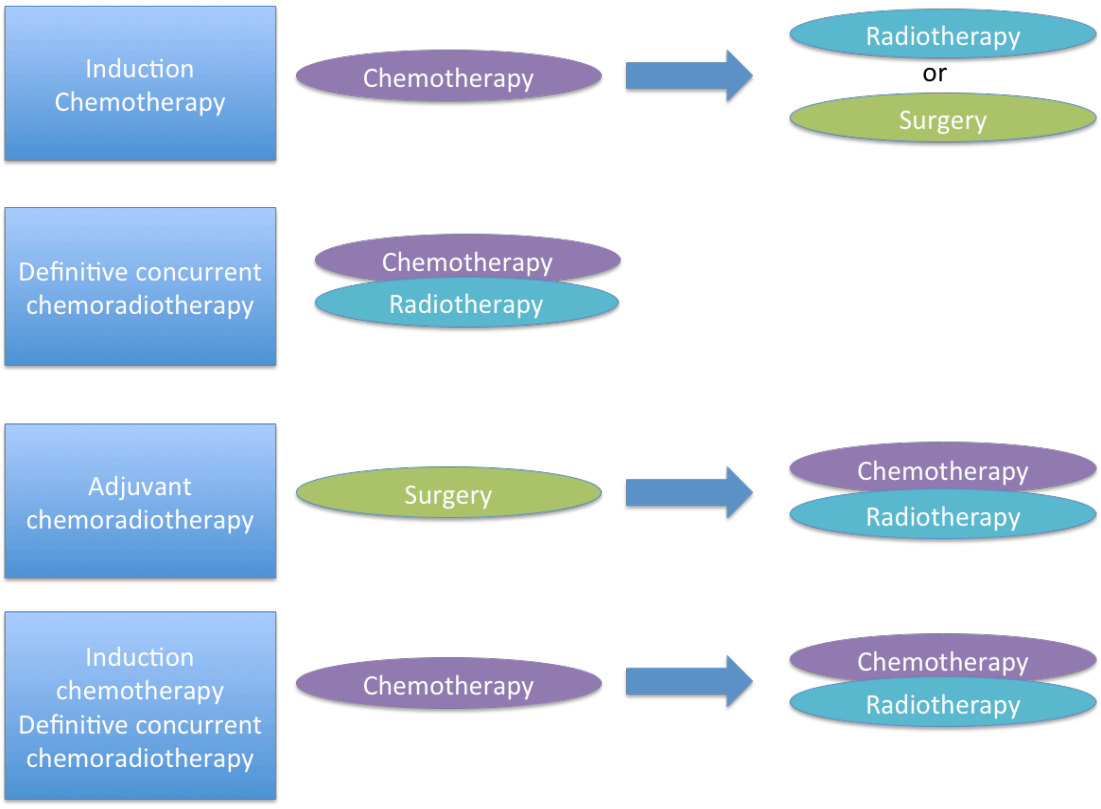



Fig 1.12 Treatment schema for loco-regionally advanced HNSCC showing possible timings of chemotherapy, adapted from (Choong and Vokes, 2008).

Historically, the most appropriate primary treatment modality for locally advanced or T3/4 HNSCC was largely determined by the tumour resectability. Although this varied between disease site and extent, surgeons and institutions, in most cases surgery was deemed the optimal modality. However, the growing amount of information derived from phase III clinical trials on the use of multimodality treatment, has lead to the questioning of the traditional surgery model.

The Department of Veterans Affairs Cooperative Studies Program (1991) began in 1984 and randomised 332 patients with resectable laryngeal SCC to receive either standard surgery followed by radiation therapy or neo-adjuvant therapy (cisplatin and 5FU) followed by radiotherapy for those achieving a greater than 50% response to the chemotherapy. Although there were fewer local recurrences and distant metastases in the chemotherapy arm, estimated two-year survival was very similar (68%) for both groups. Importantly, only 36% of patients in the chemotherapy arm required total laryngectomy (primary or salvage) within two years. These results were interpreted as suggesting that chemotherapy could be effective in avoiding laryngectomy in a large proportion of patients with advanced laryngeal cancer. However, follow-up time was relatively short (median 33 months) and there was no arm for radiotherapy alone, the effects of which may have been responsible for the laryngeal preservation.

A study by the Radiation Oncology Group and Head and Neck Intergroup (Forastiere et al., 2003) compared radiation alone with induction and synchronous chemotherapy in the treatment of stage III and IV laryngeal cancer. Median follow-up of 3.8 years demonstrated no obvious differences with regards to laryngeal preservation at this stage, but there was significantly better loco-regional control and laryngeal preservation with synchronous chemoradiation (cisplatin and 5FU).

In the year 2000, 10,741 patients from 63 high quality trials had become available and formed the basis for a large meta-analysis based upon individual patient data (Pignon et al., 2000). This study reported a 4% survival benefit at five years for chemotherapy overall: neo-adjuvant and adjuvant chemotherapy conferred

small, non-significant advantages, whilst a gain of 8% was achieved using 'concurrent' combinations. However, the authors affirmed that clear conclusions could not be drawn because of heterogeneity of the results. An update of these data in 2009 (Pignon et al., 2009), analysed 87 randomized trials including 16,485 patients with a median follow-up of 5.5 years and comprising 50 concomitant, 32 induction and 9 adjuvant chemotherapy trials. The absolute survival benefit for chemotherapy overall was 4.5% at five years and as previously reported by the group, the best results were with concomitant therapy, offering an 6.5% gain; however these latter results are free from significant heterogeneity. The least benefit was noted for adjuvant chemotherapy.

An early meta-analysis of the use of adjuvant chemotherapy in HNSCC demonstrated unfavourable results, with a survival advantage of 6% but a death rate from complications of 7% (Stell and Rawson, 1990). However, this study did not segregate tumours by staging and included studies of adjuvant, synchronous and neo-adjuvant settings.

Although the benefits of adjuvant chemotherapy are not immediately evident from the above meta-analyses, two randomised controlled trials have shown that concomitant use of cisplatin and radiotherapy after surgery increases tumour control and overall survival in high risk patients with positive resection margins or extra-capsular lymph node spread (Bernier et al., 2004, Cooper et al., 2004).

The role of induction chemotherapy, as highlighted above is controversial. Two recent randomised trials (Posner et al., 2007, Vermorken et al., 2007) suggested that the addition of docetaxel to cisplatin and 5FU given before definitive radiotherapy, improved survival. However, the use of non-standard radiotherapy and chemoradiation schedules in these trials has led to uncertainty about the benefits of this approach when standard chemoradiotherapy is prescribed, so further work is required.

A recently published Cochrane review (Furness et al., 2010) evaluated chemotherapy for the treatment of oral cavity and oropharyngeal cancer. The review included twenty-five randomised controlled trials where more than 50 percent of

participants had primary tumours in the oral cavity or oropharynx. The trials, which compared the addition of chemotherapy to other treatments such as radiotherapy and/or surgery, or compared two or more chemotherapy regimens or modes of administration, were included. The study concluded that chemotherapy, in addition to radiotherapy and surgery, was associated with improved overall survival in patients with oral cavity and oropharyngeal cancers; induction chemotherapy was associated with a 9% increase in survival and adjuvant concomitant chemoradiotherapy was associated with a 16% increase in overall survival following surgery. In patients with unresectable tumours, concomitant chemoradiotherapy showed a 22% benefit in overall survival compared with radiotherapy alone, however, the additional toxicity attributable to chemotherapy in the combined regimens remained unquantified.

In addition to traditional chemotherapy agents, biologically targeted treatment in the form of cetuximab, an anti-epidermal growth factor receptor antibody has been shown to be effective in HNSCC. The concurrent administration of cetuximab, with radiotherapy, was shown to increase overall survival (median survival 49 months versus 29.3 months) and loco-regional control in patients with HNSCC of the head and neck in a multi-centred randomised controlled trial including a total of 424 patients (Bonner et al., 2006).

In summary, the evidence for the use of chemotherapy in HNSCC is most favourable for the use of concurrent regimes. The role of induction and adjuvant therapies are less clearly understood, however it seems evident that some drug combinations for certain tumours/stage may confer a survival advantage. Clinical trials and meta-analyses largely group together tumours of several sub sites and of differing stages due to the relative rarity of some tumour sites. This highlights the potential role for methods to evaluate 'personalised' predictors of chemotherapy response.

The most commonly used drugs in clinical practice are cisplatin, 5FU, docetaxel and cetuximab, the mechanisms of which are discussed below. The local treatment guidelines in relation to dosage and timings are discussed in section 3.4.2.

### 1.7.1 Cisplatin

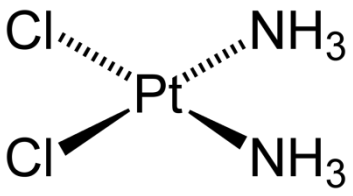



Figure 1.13 Structure of cisplatin (Rang, 2003)

Cisplatin is a water soluble square planar co-ordination complex containing a central platinum atom surrounded by two chlorine atoms and two ammonia groups (Figure 1.13). Neutrality of charge and the cis configuration are necessary for the cisplatin complex to exert anti-neoplastic activity. In the relatively high chloride concentration of plasma, the cisplatin complex is un-ionized, allowing passage of the drug through cell membranes. Intracellularly, in the presence of a low chloride concentration, the chloride ligands of the complex are displaced by water (aquation), resulting in formation of positively charged platinum complexes $[Pt(NH_3)2Cl(OH_2)]^+$ and $[Pt(NH_3)2(OH^2)2]^{2+}$ (Wang and Lippard, 2005).

The platinum complexes can react with DNA, forming both intrastrand and interstrand cross-links. The N7 of guanine is a particularly reactive site, leading to platinum cross-links between adjacent guanines on the same DNA strand (Figure 1.14). DNA adducts formed by cisplatin inhibit DNA replication and transcription and lead to breaks and miscoding. This damage activates several signal transduction pathways, including those involving ATR, p53, p73, and MAPK, and culminate in the activation of apoptosis (Siddiq, 2003).

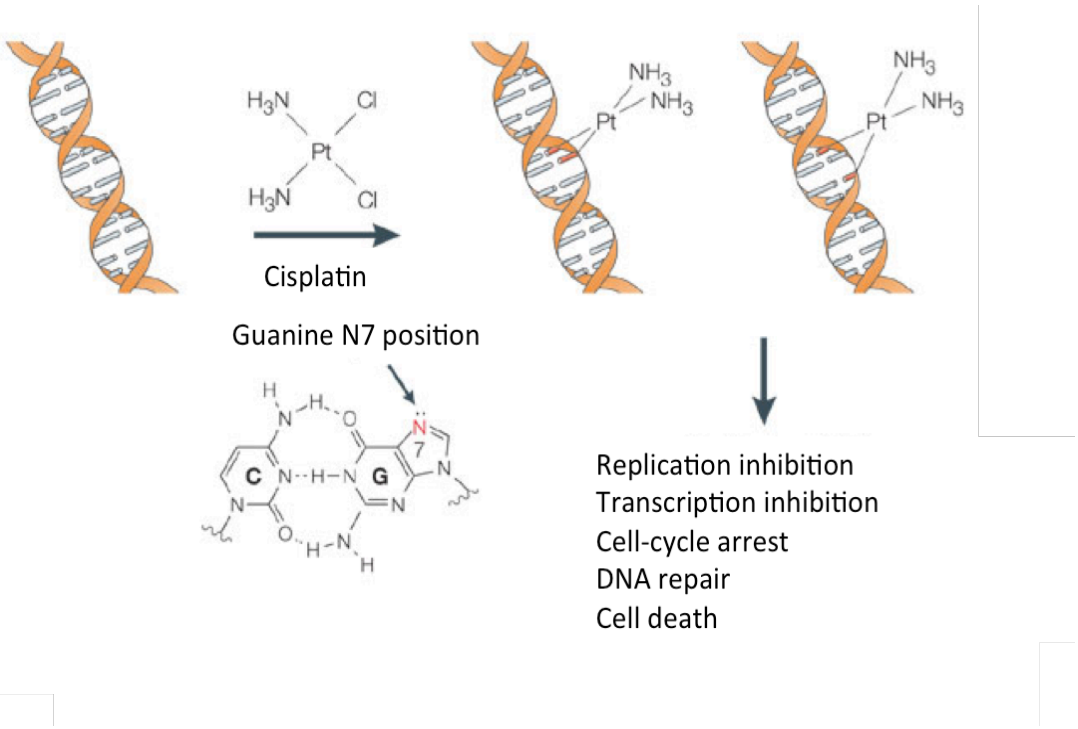



Figure 1.14 Diagram depicting the platinum atom of cisplatin binding covalently to the N7 position of purines to form 1,2- or 1,3-intrastrand crosslinks, and interstrand crosslinks to bring about various cellular responses (Wang and Lippard, 2005).

Cisplatin resistance is a common problem in clinical practice with several potential mechanisms implicated. These include reduced drug accumulation due to cell membrane barriers (Timmer-Bosscha et al., 1992), enhanced repair of cisplatin DNA adducts or enhanced capacity to tolerate cisplatin-induced damage due to alterations in proteins that recognise cisplatin-DNA damage (mismatch repair and high-mobility group (HMG) family proteins (Perez, 1998)).

The altered expression of oncogenes and tumour suppressor genes are also important contributors to cisplatin resistance. Considerable evidence indicates that mutated p53 plays a significant role in the development of cisplatin resistance since several genes implicated in drug resistance and apoptosis (e.g. mismatch repair, bcl-2, HMGs, DNA polymerases alpha and beta, PCNA, and insulin-like growth factor) are known to be regulated by the p53 oncoprotein (Dempke et al., 2000). By using p53 genetic suppressor elements to decrease protein levels, Gallagher et al. (1997) saw an 8-fold increase in cisplatin resistance in ovarian cancer cells. Within HNSCC cell lines, wild-type p53 tends to confer cisplatin resistance. Bradford et al. (2003)

identified mutations of the p53 gene in 13 of 23 HNSCC cell lines tested. The average drug dose required to inhibit 50% of cell growth for cell lines with mutant p53 was 6.8 μmol compared with 13.7μmol for cell lines with wild-type p53.

Cisplatin resistance has been shown to correlate with *c-myc* dependent expression of cyclin D1 (Warenius et al., 1996), with carcinoma cell lines expressing higher levels of cyclin D1 exhibiting higher resistance to cisplatin. The association of loss of p16 expression with poorer outcome in HNCSC patients as previously described, in some circumstances may be due to the reduced cisplatin-induced cell cycle arrest through the p16/p53-dependent pathway (Yip et al., 2006). In contrast, Taguchi et al. (2004) found HNSCC cell lines with strong expression of p21, p27 or Bax showed significantly higher sensitivity to cisplatin as opposed to other commonly administered chemotherapy agents used in the treatment of HNSCC.

The role of p53 and p16 in the cytotoxic action of cisplatin is of potential clinical importance as these genes may have significance as prognostic markers to predict a tumour's chemosensitivity. Yip et al. (2006) demonstrated the elevated expression of p16, p53 and p21 following the addition of cisplatin by western blot analysis in parental and antisense cyclin D1 cell lines, and suggested that the evaluation of the expression status or indeed changes in expression of p53 and p16 following chemotherapy treatment *in vitro* may be a valuable tool for treatment planning.

### 1.7.2 5-Fluorouracil

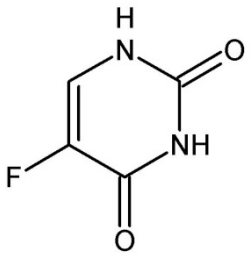



Figure 1.15 Structure of 5-fluorouracil (Rang, 2003).

5FU is a pyrimidine analogue which inhibits the synthesis of DNA via its interference with thymidylate synthesis (Rang, 2003). It is converted into a

fraudulent ‘nucleotide’ fluorodeoxyuridine monophosphate (FdUMP) that interacts with thymidylate synthetase and the folate cofactors. As this cannot be converted into thymidylate (as in FdUMP the fluorine has replaced hydrogen at C5 where methylation would take place), this carbon-fluorine bond is less susceptible to enzymatic cleavage than the carbon-hydrogen bond. This results in inhibition of DNA synthesis but not RNA and protein synthesis.

As with cisplatin, resistance to 5FU is commonly encountered and is multifactorial. Possible pathways include transport mechanisms, increased metabolism, protection from apoptosis, and resistance via cell cycle kinetics (Mader et al., 1998).

Regarding the effect of 5FU on the cell cycle in HNSCC, Liu et al. (2006) found that 5FU induces apoptosis via a p53-independent pathway; p53 expression was not induced in either of their laryngeal cell lines tested. 5FU induced the accumulation of Rb protein and a cyclin dependent kinase inhibitor, p21WAF1/CIP1 and in addition, G1/S cell cycle phase arrest was associated with anti-proliferative activity of 5FU in both cell lines. Conversely, Lee et al. (2005) found in a panel of hypopharyngeal carcinoma cell lines, that following exposure to 5FU, p21WAF1/CIP1 protein and p53 protein increased, and G1-phase arrest of the cell cycle was observed whereas apoptosis was not, suggesting that the mechanism is not fully understood or may differ between HNSCC tumour sub sites.

### 1.7.3 Docetaxel

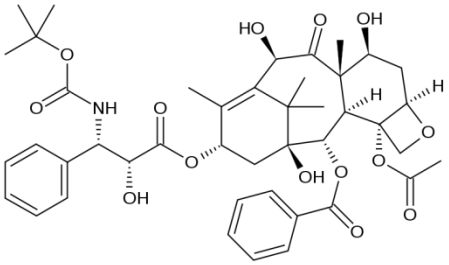



Figure 1.16 Structure of docetaxel

Docetaxel belongs to the taxane group and is a semi-synthetic analogue of paclitaxel. Docetaxel reversibly binds to microtubules with high affinity resulting in stabilisation of the microtubules. The resultant reduction in the free tubulin needed for microtubule formation prevents mitotic cell division between metaphase and anaphase (Eisenhauer and Vermorken, 1998). Additionally, because microtubules do not disassemble in the presence of docetaxel, they accumulate inside the cell and cause initiation of apoptosis. Apoptosis is also encouraged by docetaxel blocking (via phosphorylation) the 'apoptosis inhibiting' oncoprotein bcl-2.

The development of acquired resistance to docetaxel often occurs; several possible mechanisms have been shown, including altered expression of mRNA β-tubulin isotypes and modulation of β-tubulin protein levels (Shalli et al., 2005) high expression of P-glycoprotein (Bissery et al., 1995), reduced expression of p27 (Brown et al., 2004) and high expression of thioredoxin (Kim et al., 2005). However, the mechanisms involved are not yet completely understood.

With regards to predictors of sensitivity, Taguchi et al (2004) demonstrated HNSCC cells with strong expression of Bax or weak expression of cyclin E showed significantly higher sensitivity to paclitaxel. Furthermore Kawakami and colleagues showed that Bax expression changed from negative to positive in one HNSCC cell line and Bcl-2 expression decreased in another following incubation with paclitaxel, leading the authors to conclude that Bcl-2/Bax status was correlated with drug sensitivity (Kawakami et al., 1999).

### 1.7.4 Cetuximab

Cetuximab is a chimeric (mouse/human) monoclonal antibody against the ligand-binding domain of the epidermal growth factor receptor (EGFR). The EGFR is a member of the HER family of tyrosine kinase growth factor receptors, a group of proteins whose aberrant activity plays a key role growth and metastasis of solid tumours (Bernier, 2006). Binding to EGFR by its endogenous ligands, e.g. epidermal growth factor (EGF) or transforming growth factor (TGF-α), results in stimulation of downstream signal-transduction pathways (Figure 1.17) such as the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, and signal transducers and activators of transcription (STAT) signalling pathways, ultimately leading to cell cycle progression, reduced apoptotic capacity, angiogenesis, and metastatic phenotypes (Burtness, 2005).

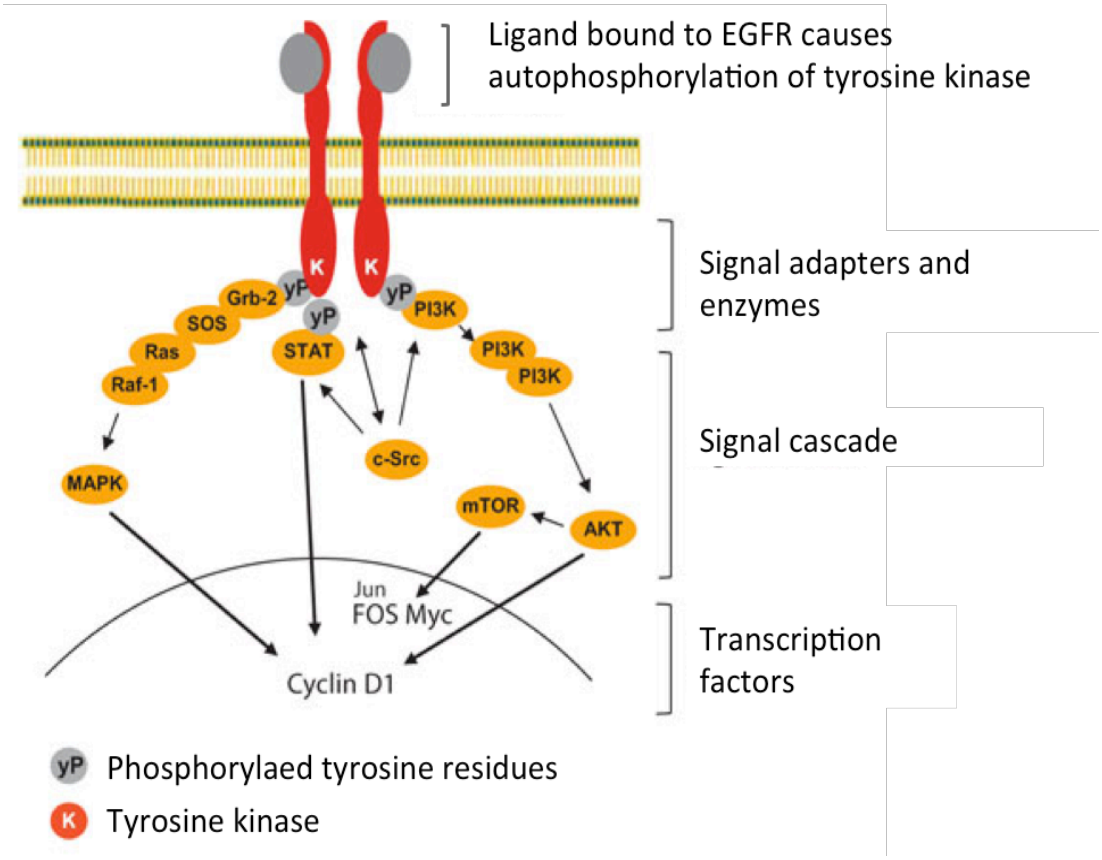



Figure 1.17 Diagram of he EGFR signalling pathway. Adapted from (Lurje and Lenz, 2009).

Cetuximab binds competitively to the EGFR, preventing dimerization and activation of the intrinsic receptor tyrosine kinase (Goldstein et al., 1995). Cetuximab also affects ligand-independent EGFR signalling (Dittmann et al., 2005). In preclinical models, treatment with cetuximab stimulated EGFR internalization/degradation (Prewett et al., 1996), enhanced tumour-cell apoptosis, and inhibited proliferation, angiogenesis, and invasiveness (Huang et al., 1999, Saleh et al., 1999, Milas et al., 2004).

EGFR and its ligand TGF-α are over-expressed in nearly all head and neck tumours ( Christensen et al., 1992, Grandis and Tweardy, 1993) and over-expression is associated with greater radioresistance/chemoresistance, shorter disease-free and overall survival in patients (Grandis and Tweardy, 1993, Ang et al., 2002, Liang et al., 2003, Hitt et al., 2005, Pivot et al., 2005,). The EGFR appears to be specifically associated with adaptive responses to radiation; after exposure to radiation, the EGFR activation observed in radio-resistant tumour models is greater than in tumours that originally were radiosensitive (Milas et al., 2000). Blockage of EGFR has been shown to increase cell sensitivity to radiation (Bonner et al., 1994).

In the clinical management of HNSCC, cetuximab is predominantly used concomitantly with radiotherapy due to its radio-sensitising actions, rather than as a cytotoxic agent *per se*, as highlighted by the findings of Bonner et al (2006) described in section 1.7. The current National Institute of Clinical Excellence guidelines state that cetuximab, in combination with radiotherapy, is recommended as a possible treatment for people with locally advanced squamous cell cancer of the head and neck only if they have a Karnofsky performance-status score of 90% or more, and all forms of platinum-based chemotherapy are considered inappropriate (http://www.nice.org.uk/TA145).

### 1.7.5 Predicting Chemotherapy Response

Given the increasing availability of new treatment strategies such as induction chemotherapy, concurrent and sequential chemo-radiation concepts, and multimodality-based organ preservation strategies (e.g. IMRT), treatment planning with regard to optimal outcome is becoming increasingly challenging. Chemo-

radiation is not effective in all patients and when used unsuccessfully, patients suffer the potential side effects and toxicities of chemotherapy (e.g., swallowing problems, hearing loss) and radiation therapy (e.g., mucositis, late toxicity). Moreover, the outcome is difficult to predict due to heterogeneity of a tumour's response, impaired late functional outcome and increased late toxicity if simultaneously applied to radiation. The identification of reliable outcome predictors in this setting is of great interest and of particular importance in advanced HNSCC where a range of treatments, namely chemotherapy, surgery, post-operative radiotherapy and cetuximab with radiotherapy are available.

A consensus document by Lefebvre and Ang (2009), outlining the guidelines for the conduct of future phase III clinical trials of larynx preservation in patients with locally advanced laryngeal and hypopharyngeal cancer, emphasised the importance of translational research in HNSCC. They concluded that future studies should include a comprehensive evaluation of the head and neck cancer molecular signature for correlation with treatment response, toxicity, and survival. The authors, acknowledging the importance of predicting response to chemotherapy, suggested that further work into the role of biomarkers in HNSCC was warranted, based on early evidence with certain markers that have demonstrated prognostic value and/or a potential role for guiding treatment decisions. Over-expression of EGFR is well documented as described in section 1.7.4. Additional biomarkers of interest in the literature include E-cadherin and β- catenin (markers for epithelial-to-mesenchymal transition (Khambata-Ford et al., 2007, Mandal et al., 2008), epiregulin and amphiregulin (markers for response to EGFR antagonists in colorectal carcinoma) (Khambata-Ford et al., 2007) and p53 mutation (Poeta et al., 2007). Furthermore, chemo-resistance of tumour cells, in general, is associated with mutations in oncogenes (e.g., K-ras), loss of tumour suppressors (e.g., p53, p16) or dysregulation of genes involved in cell cycle control, cell proliferation, signal transduction, angiogenesis, or apoptosis (Glinsky, 2008). However, in HNSCC these biomarkers only show weak correlation with improved outcome after chemo-radiation (Hoffmann et al., 2008) and clinical relevance is still limited (except regarding p16INK4a and HPV-16 in oropharyngeal cancer as described in sections 1.3.2.6 and 1.3.3).

There is an increasing availability of literature centred on assay-based chemo-response evaluation in individual tumours. In principle, assays can be divided into 2 groups: clonogenic (first expanding tumour cells before treatment with cytotoxic agents) and non-clonogenic (primary tumour cells are exposed to chemotherapy drugs). The read-out of the assays is commonly the measurement of cell proliferation, cell death or metabolic activity. Clonogenic assays have been shown to be clinically predictive in ovarian cancer, as demonstrated by Gallion et al. (2006) using an *ex vivo* chemo-response assay (ChemoFX) for uncovering chemo-resistance. Nevertheless, the major clinical need, i.e., the reliable determination of individual chemosensitivity patterns of solid tumours by means of *ex* vivo assays is still unmet (Dietz et al., 2010).

Although *in vitro* based assays represent an alternative, Miyamoto et al. (2004) highlight the difficulties with using such methods. Such techniques fail to provide a functional tumour microenvironment (i.e., extracellular matrix, blood vessels, nerves, and soluble factors produced by leukocytes or the tumour itself) and can also result in clonal expansion of subpopulations of tumour cells not representative of the tumour as a whole, in cell culture. These factors, combined with the possible absence of immune cells, may influence the outcome and cause differences to the clinical response to any given chemotherapy.

For individual chemosensitivity of solid tumours (e.g. HNSCC), assays have to meet certain criteria to be useful in terms of clinical practicability and methodical accuracy. The qualities of such an assay have been suggested by Dietz et al. (2010):

1) ability to determine drug responses in small amounts of

tumour tissue, mostly from tumour biopsies

2) robust standard operating procedures for harvesting, handling, and processing tumour samples

3) methodical solutions to exclude reactions in non-malignant tumour cells (stroma) reliably

4) high feasibility

5) high predictive accuracy

6) provision of results in a clinically adequate time range

7) quantatively reliable information on drug response

As will be discussed in greater detail below, micro fluidic devices possess the majority of these characteristics, providing an *in vitro* platform for evaluating chemotherapy response in HNSCC.

## 1.8 HNSCC Culture Techniques

The ability to develop continuous cell lines from human tumours (e.g. HeLa; (Gey, 1952), aided by the studies of Hayflick on the finite life span of cells in culture (Hayflick, 1961) led to the rapid expansion in the use of cell lines in the study of intracellular activity and flux, environmental and cell-cell interactions, genetics and cell products and secretions. Following the discovery that head and neck cancer lines could be successfully cultured *in vitro* (Easty et al., 1981, Krause et al., 1981, Rheinwald and Beckett, 1981), a variety of cell, tissue and organ culture techniques have been applied to its study.

The loss of histological characteristics seen in primary and disaggregated cell line models has led to the development of culture techniques aimed at the preservation of tissue architecture and maintenance of cell-cell and cell-matrix interactions. Numerous three-dimensional culture techniques have been developed and can be divided into, a) organ culture utilising the whole or small fragments of the original tissue, b) histotypic culture, growing cell lines at high density in a three dimensional matrix and c) organotypic cultures, the maintenance of different cell lineages, present in *in vivo* ratios and spatial relations (Figure 1.18).

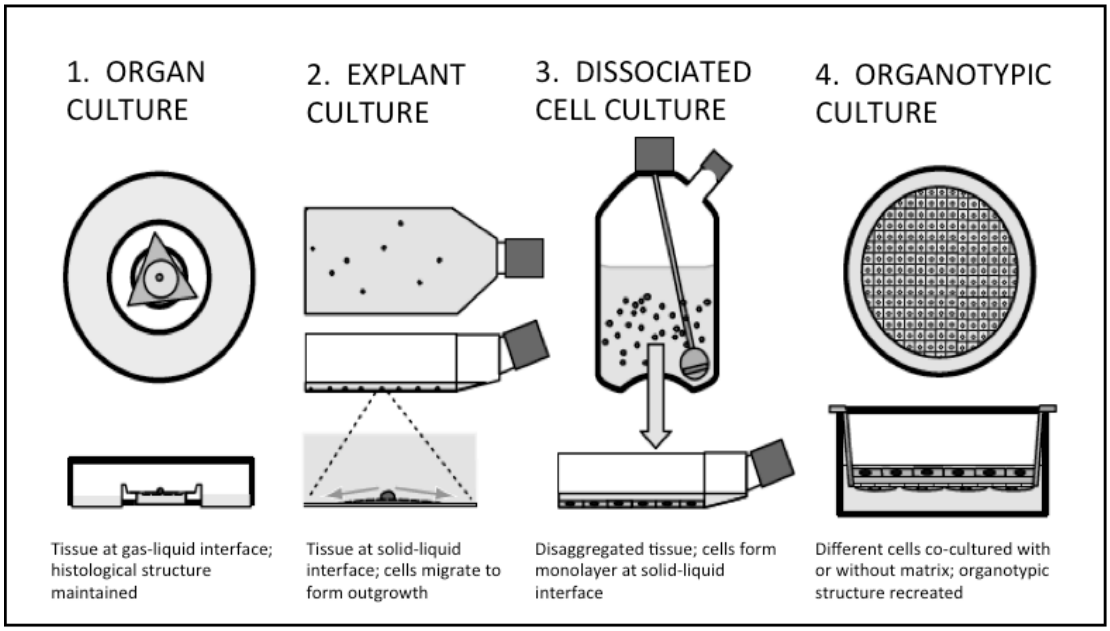



Figure 1.18 Diagram of different tissue culture methods, 1) Organ culture on a filter disk on a triangular stainless steel grid over a well of medium, 2) Explant cultures in a flask, 3) Disassociated cell culture in a stirred vessel with an enzymatic disaggregation generating a cell suspension seeded as a monolayer in the lower diagram, 4) Organotypic culture, a filter well showing an array of cells, seen in section in the lower diagram, combined with matrix and stromal cells (Vunjak-Novakovic and Freshney, 2006).

### 1.8.1 Cell culture

Cells isolated from donor tissue may be maintained in a number of different ways and may be categorised into either 1) primary explant culture or 2) dissociated cell culture. The spontaneous or mechanical adherence of a fragment of tissue can produce an outgrowth of cells; the primary explant, with the migrating cells termed the outgrowth. Cells in the outgrowth are selected, firstly, by their ability to migrate from the explant and subsequently, if sub cultured, by their ability to proliferate. A sample of tissue may also be mechanically or enzymatically disaggregated; cells and small aggregates capable of attachment to a solid substrate then forming a monolayer. Cells within the monolayer capable of proliferation will then be selected

at the first subculture and, as with the outgrowth from a primary explant, may give rise to a cell line. By disaggregating tissue, larger, more rapid cultures are produced as compared to explant culture, however explant culture may still be preferable where only small fragments of tissue are available or the fragility of the cells precludes survival after disaggregation. Generally, the differentiated cells in a tissue have limited ability to proliferate and do not therefore contribute to the formation of a primary culture, unless conditions are adapted to promote their attachment and preserve their differentiated status. It is therefore the proliferating committed precursor cells that give rise to the primary culture (Figure 1.19).

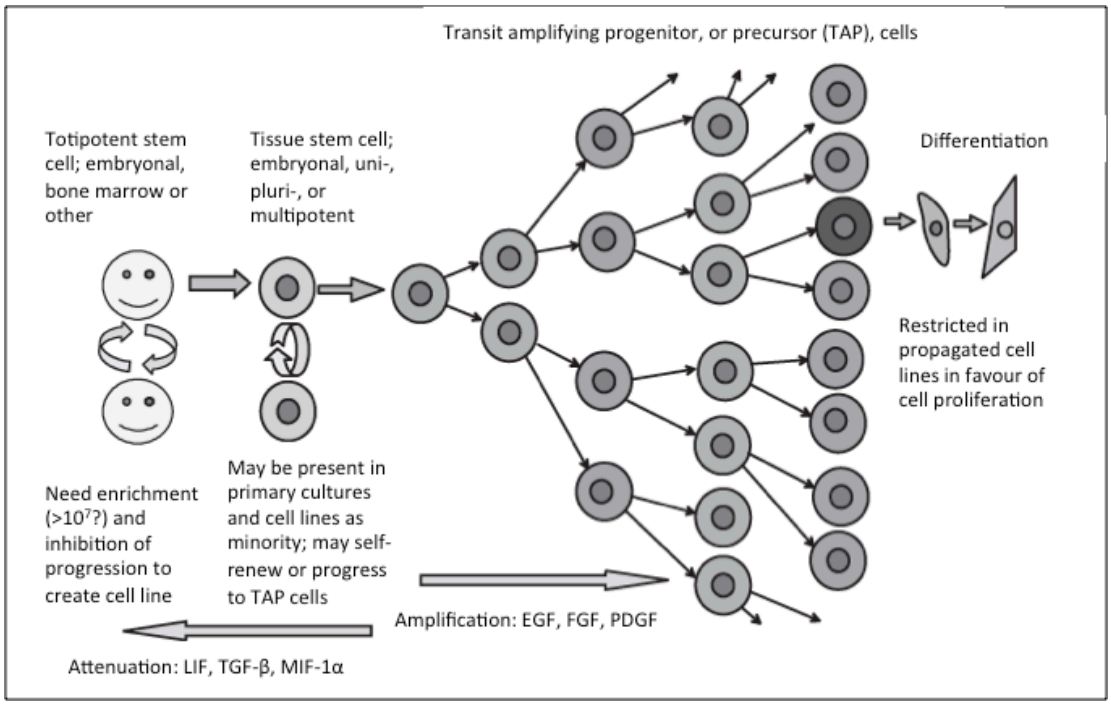



Figure 1.19 Diagram demonstrating the origin of cell lines; progression from totipotent stem cell, through tissue stem cell (single or multiple lineage committed), to transit amplifying progenitor cell compartment. LIF; leukaemia inhibitory factor, TGF-β; transforming growth factor β, MIF-1α; Macrophage migration inhibitory factor 1-alpha, EGF; epidermal growth factor, FGF; fibroblast growth factor, PDGF; platelet derived growth factor. Adapted from Freshney (2005).

Immortalized cell lines derived from HNSCC have proved invaluable as tools in researching the molecular, biochemical, genetic and immunological properties of

HNSCC. Advantages of using cell lines include ease of use, rapid growth, result reproducibility (Cree et al., 2010), reduced cost and avoidance of legal and ethical issues associated with animal experimentation (Lin et al., 2007). The establishment of head and neck cell lines, like other solid tumours, was initially hampered by fibroblast overgrowth, long quiescent periods before the cells can be sub cultured, and the dependence on feeder layers in primary and secondary cultures (Moore et al., 1955, Easty et al., 1981, Rheinwald and Beckett, 1981). However with technical improvements, the permanent culture of HNSCC cell lines has been successful, Lin et al. (2007) reporting the establishment of over 300 cell lines in 2007.

The basis for using a cell line is the belief that it will mimic the clinical situation. It is known however that tumours are not composed of layers of homogenous cells but rather areas of heterogeneity exist within the tumour mass, containing differing genetic expression and biological properties. As described above, cell culture is a selective process; cells must possess the potential to grow in culture medium, within which they experience a very different environment to that of their originating tumour. The cell culture environment, namely substrate, gas composition and medium can have significant influence on the characteristics of cells that propagate from clinical samples. Ince et al. (2007) found that by adapting conditions for the culture of primary breast epithelial cells (using a novel serum free media and differing culture ware) cell cultures formed were more tumourigenic, formed xenografts that more closely represented breast ductal adenocarcinoma and had greater metastatic potential when transplanted into animals.

Homogenous cell lines also lack the extra-cellular matrix containing proteins, such as collagen, elastin and laminin, that give the tissue their mechanical properties and help to organise communication between cells embedded in the matrix e.g. via adherence by the integrin family of cell receptors. Lack of cell-cell contact, namely the absence of cell-mediated signalling, formation of junctional complexes and reduced potential for exchange of homocrine factors is apparent in cell lines (Freshney, 2005). This effect is manifested as cessation of cell motility and withdrawl from the cell cycle in normal cells and reduced proliferation and increased apoptosis in transformed cells. The interaction of different populations of cells is also often key

in maintaining functionality of another cell type. Alveolar cells of the lung, for example, only produce and release surfactant in response to the hormonal signalling from adjacent fibroblasts (Post et al., 1984).

Lack of heterogeneity also results in the absence of cells corresponding to cancer stem cells. The theory of cancer stem cells states that a small subset of cancer cells within the tumour mass has the exclusive capacity to both divide and expand the cancer stem cell pool and to differentiate into non-tumourigenic, more differentiated cancer cell lineages (Cho and Clarke, 2008). This concept was first demonstrated in a study of leukaemia where only cells with specific surface antigen profiles were able to cause leukaemia when engrafted into immunodeficient mice (Lapidot et al., 1994). This theory has been more recently extended to solid tumours including HNSCC, in which a minority population of CD44+ cancer cells, which typically comprise <10% of the cells in a HNSCC tumour, gave rise to new tumours *in vivo* and possessed both ability to self-renew and to differentiate (Prince et al., 2007). Such cells *in vivo* are more highly resistant to chemotherapy and are postulated to be responsible for the recurrence of cancer after treatment. Behavioural heterogeneity of adherent cell lines derived from gliomas and capable of initiating xenograft tumours suggests that the diversity of these tumours may reflect differing stem cell phenotypes (Pollard et al., 2009).

Within tumours, malignant cells *in vivo* are normally capable of independent growth and show reduced attachment to substrate (usually basement membrane proteins). In contrast, cell lines commonly become dependant on growth factors supplied as serum or other supplements and adherence to plastic. Withdrawal of serum or use of plastics such as polypropylene, which do not encourage cell adherence result in the death of many cell lines (Cree et al., 2010). This again demonstrates the limitations of translating information derived from of cell line studies to *in vivo* tumours.

Cell line cultures generally exhibit a much greater rate of growth than their parent tumours. Whilst this is useful to the researcher in terms of reducing the time

needed for establishment of cells for experimentation, the variable S-phase fractions and doubling times witnessed *in vivo* are not correctly represented.

Cell lines have contributed greatly to cancer research due to their ease of availability, use and general reproducibility. However their failure to adequately replicate *in vivo* characteristics limits their clinical applicability and has therefore led to an interest in alternative culture techniques.

### 1.8.2 Organ Culture

Organ culture allows for the maintenance of the normal, *in vivo* histo-architecture and diversity of cell types. However, the absence of a functional vascular system severely limits the size of biopsy that can be maintained alive, by diffusion, as lack of nutrient supply and gas exchange of the cells becomes limited, ultimately leading to central necrosis (Freshney, 2005). Retention of histological structure and associated differentiated properties may be enhanced at the air/medium interface, where gas exchange is optimized and cell migration minimised.

In order to optimize nutrient and gaseous exchange, supporting materials are used to keep the tissue at the gas interface. Various methods have been described, including semi-solid agar gel (Wolff and Haffen, 1952), clotted plasma (Fell and Robison, 1929), micropore filter, lens paper, or strips of Perspex or Plexiglass. The organ cultures can also be grown on top of a stainless steel grid (Freshney, 1992). Another popular choice for growing organ cultures is the filter-well inserts. Filter-well inserts with different materials like ceramic, collagen, nitrocellulose are now commercially available and have been successfully used to develop functionally integrated thyroid epithelium, stratified epidermis, intestinal epithelium, and renal epithelium (Freshney, 2005). Although anchorage to a solid substrate can lead to the development of an outgrowth of cells from the explant, this can be minimised if a non-wettable substrate is used and if the level of the gas-liquid interface is kept optimal.

Organ culture has been used with some success in HNSCC. The culture of oral mucosa has been studied (MacCallum et al., 1987) and information regarding

interactions between tumour and basal epithelial cells derived from studying the effects of differing calcium concentrations on dissociation of upper layers of oral mucosa (Sacks et al., 1985) used to propose models of invasion. Kleinsasser et al. (2004) used mini-organ cultures of human UADT epithelia to study the resultant DNA damage caused by exposure to genotoxic compounds *in vitro.* The organ culture size was however limited to $1mm^3$. Using a similar technique (1 $mm^3$ biopsies in 24-well plates coated with agarose gel), Baumeister et al. (2009) studied the impact of stimulation by TGF-α on carcinogen-induced and oxidative DNA damage in mucosa tissue cultures of macroscopically normal biopsies from tumour patients and controls. They demonstrated a DNA-stabilizing effect of stimulation by TGF-α in mucosa tissue cultures of tumour patients suggesting this as a physiological response to continued carcinogenic impact on the epithelium of the UADT.

Organ culture is an expensive, labour intensive technique that offers little reproducibility and is dependant on fresh donor tissue as organ cultures are unable to be propagated beyond a number of weeks at most. As organ cultures are made up of mostly fully differentiated cells, cultures do not grow, only the outer layers of cell, at the air/medium interface where conditions are more favourable for growth, may demonstrate evidence of proliferation. The problem of heterogeneity between cultures is also recognised; the heterogenous histo-organisation of a tumour is well known and if one considers the dissection of such tumours into sub millimetre sized pieces, differences between individual organ cultures are easily understood (Sacks, 1996).

### 1.8.3 Histotypic Culture

Histotypic cultures aim to recreate the density of cells found in the original tissue and include such techniques as simply allowing the cells to multilayer by perfusing a monolayer (Kruse et al., 1970), recreating a highly complex perfused membrane (Klement et al., 1987), the creation of capillary beds (Knazek et al., 1972) and the development of spheroids (Linser and Moscona, 1979).

To address the issue of limited gaseous and nutrient transfer at high cell densities, Knazek et al. (Knazek et al., 1972) developed a perfusion chamber from a

bed of plastic capillary chambers that are gas and nutrient permeable whilst able to support cell growth on their outer surface. There is evidence that this goes some way to the recreation of the tumour microenvironment. For example colonic carcinoma cells have been shown to produce elevated levels of carcinoembryonic antigen when cultivated in hollow fibres as compared with a monolayer culture (Rutzky et al., 1979, David et al., 1978) with comparable molecular properties to those found *in vivo*.

Spherical aggregates of malignant cells, i.e. multicellular tumour spheroids (MTS), have also been proposed as *in vitro* models of tumour microregions and of an early, avascular stage of tumour growth. The growth of MTS can be initiated either by stimulating single cells to generate spherical colonies directly through proliferation, or by initially inducing cell aggregation with subsequent growth of the aggregates (Mueller-Klieser, 1987). More recently, microtechnologies in the form of 3D microwells and planar micropatterns have been used to provide the same cell aggregation conditions, but unlike the macroscale approaches above, these technologies offer the ability to replicate cellular and tissue parameters precisely, in order to mass produce uniform tumour spheroids.

Spheroids have the ability to mimic some *in vivo* tumour properties such as volume growth kinetics, cellular heterogeneity, e.g. the induction of proliferation gradients and quiescence, as well as differentiation characteristics, such as the development of specific histological structures or the expression of antigens (Mueller-Klieser, 1987). The technique of experimentally inducing aggregates of animal cells dates to the studies on morphogenesis in amphibian embryos by Holtfreter (1944) and was applied to the study of tumour (melanoma) cells by Moscona (1957).

Cancer cells in spheroids are exposed to a tumour-like microenvironment, mainly influenced by the distance of cells from the spheroid surface. This situation is possibly equivalent to that of cancer cells *in vivo* located at different distances from a vessel supplying nutrients. The supply conditions for microregions within a tumour may vary to a large extent, leading to pronounced heterogeneities in relevant supply

parameters such as local oxygen tensions (*p*O2), glucose concentrations or pH (Vaupel et al., 1981). These pathophysical gradients are summarised in Figure 1.20. Most notable is the central area, with reduced $O_2$ tension and lacking in aerobic metabolism (ATP), and evidence of necrosis both histologically and by immunohistochemical analysis. As with organ cultures, the growth of spheroid cultures is limited by diffusion with maximal growth of about 1000μm (Sutherland, 1988), with a typical secondary central necrosis usually established from 500μm (Hirschhaeuser et al., 2010).

Table 1.6 Table summarising research involving the therapeutic applications of tumour spheroids. HUVEC; human umbilical vein endothelial cells, BCNU; 1,3-bis(2-chloroethyl)-l-nitrosourea , GFP; green fluourescent protein, MIBG; met-aiodobenzylguanidine , MCTS; multicellular tumour spheroids, VEGF; Vascular endothelial growth factor, CTL/LAK; cytotoxic T lymphocyte/human lymphokine activated killer cells, PBMC; peripheral blood mononuclear cell, EMD 72000; humanised anti-EGFR monoclonal antibody matuzumab, DC; dendritic cells, TAA; tumour-associated antigen.

| *3D Model* | *Cell types* | *Application/therapy* | *Reference* |
|---|---|---|---|
| Mixed Spheroids | HNSCC cell line + fibroblasts | Anti-EGFR monoclonal antibody | Hoffmann et al. (2009) |
| | Prostate cancer cell line +HUVEC+ osteoblasts | Micro fluidic system for growth analysis | Hsiao et al. (2009) |
| | Cervical cancer line + fibroblasts | Survival after radiotherapy | Djordjevic and Lange (2006) |
| | Mouse fibrosarcoma line (cisplatin sensitive and resistant) | Survival after radiotherapy | Frenzel et al. (1995) |
| | Rat brain tumour cells (sensitive and resistant to 1,3-bis(2-chloroethyl)-l-nitrosourea) | BCNU treatment | Tofilon et al. (1987) |
| (Transfectant) Mosaic spheroids | Glioma cell line transfected with noradrenaline transporter gene or GFP | β-emitting radio- pharmaceutical [131I] MIBG to assess bystander effects | Boyd et al. (2002) |
| MCTS on monolayer | Colon carcinoma cells on monocytes/fibroblasts | Cathepsin B expression, invasiveness | Krueger et al. (2005) |

| | | | |
|---|---|---|---|
| MCTS+ immune cell suspension | Melanoma cell lines on endothelial cell lines | Role of T-cadherin in tumour angiogenesis | Ghosh et al. (2007) |
| | Benign/malignant urothelial cell line +monocytes/macrophages | Influence of cytokines on monocyte to macrophage differentiation | Konur et al. (1998) |
| | Breast cancer cell line + macrophages | VEGF expression and *de novo* vessel formation | Bingle et al. (2006) |
| | EC from benign/malignant pancreatic tissue endothelial + T cells/Treg | T cell/Treg infiltration studies | Nummer et al. (2007) |
| | Breast cancer cell line + monocytes | Monocytes as vehicles for gene therapy | Muthana et al. (2008) |
| | Bladder/renal carcinoma cell line + CTL/LAK | Assess number of T cells for adoptive therapy | Kawai et al. (2001) |
| | HNSCC cell line +PBMC | Therapeutic antibody EMD 72000 | Hoffmann et al. (2009) |
| | HNSCC cell line +PBMC | Trifunctional bispecific antibody catumaxomab | Hirschhaeuser et al. (2009) |
| | Urothelial/prostate carcinoma, melanoma cell lines +monocytes | Differentiation of monocytes to DC | Hirschhaeuser et al. (2009) |
| | Diverse entities + CTLs | Cytotoxicity of CTLs | Fischer et al.(2007) |
| | Melanoma cell line + CTLs | TAA recognition by CTLs | Feder-Mengus et al. (2007) |

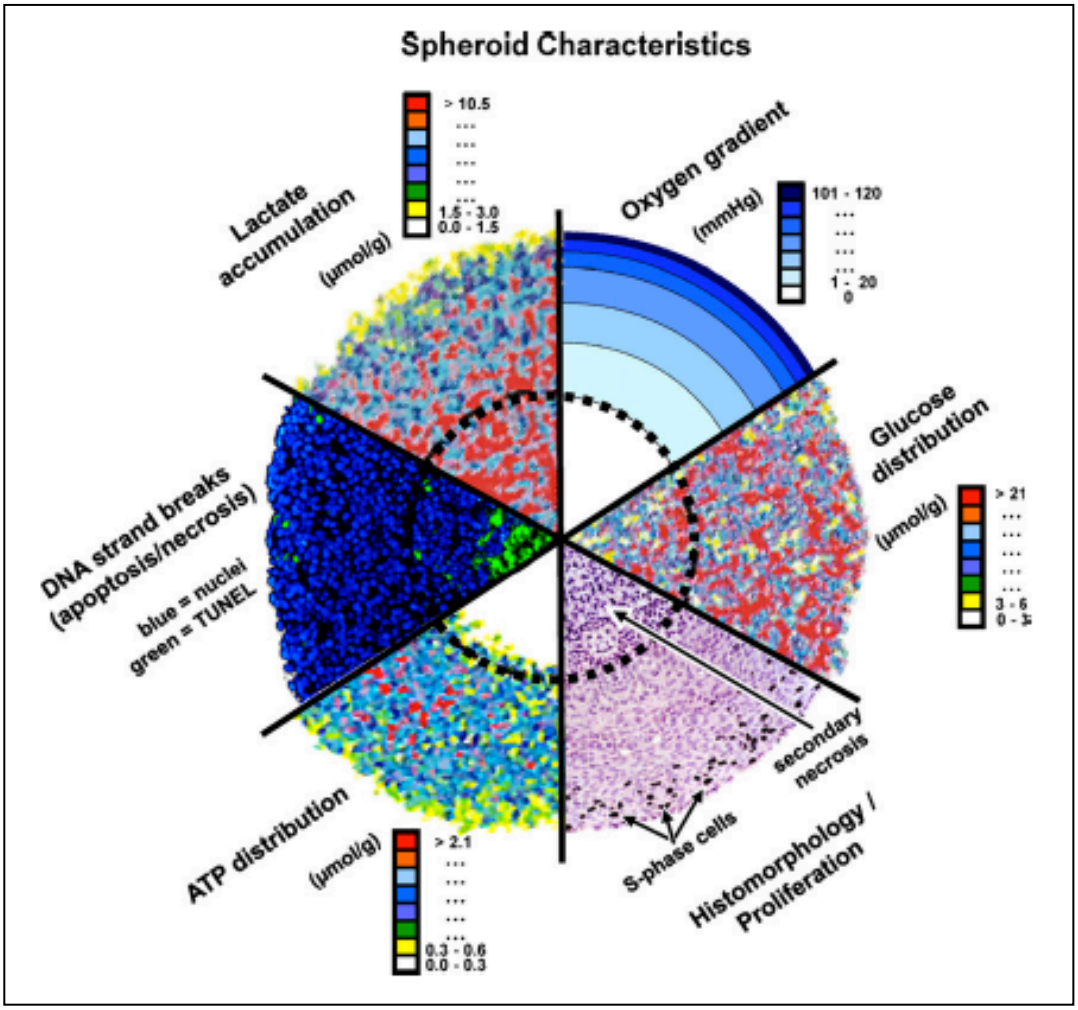



Figure 1.20 Diagram depicting the combination of analytical images of spheroid median sections studied with different technologies: autoradiography, the TUNEL assay, bioluminescence imaging and probing with oxygen microelectrodes. Together these measurements enable the concentric arrangement of cell proliferation, viability and the micromilieu in large spheroids to be understood (Hirschhaeuser et al., 2010).

Another method of creating a three-dimensional tumour model based on isolated cells or cell lines is by implanting cells in a 3D matrix scaffold generated from either purified molecules or synthetic materials, following the demonstration that both normal and malignant cells penetrate cellulose sponge (Leighton et al., 1968) facilitated by collagen coating. Cells can be implanted within a 3D matrix scaffold generated from either purified molecules, such as collagen type I, or

synthetic materials, such as cross-linked glycosaminoglycan composites (Fischbach et al., 2007, Yamada and Cukierman, 2007). Another class of 3D matrices includes those generated by cells *in vitro*, in which fibroblasts are stimulated with ascorbic acid to increase their collagen production followed by extraction of fibroblasts (Bass et al., 2007, Caswell et al., 2008, Cukierman et al., 2001). The advantage of these 'cell-derived matrices' is the use of the naturally deposited matrix as well as the ability to visualize cells using live-cell imaging. However, these matrices often have lower amounts of collagen, larger internal spaces and less depth than mature tissue matrices, making them less suitable for the study of invasion (Even-Ram and Yamada, 2005, Yamada and Cukierman, 2007). Three-dimensional cell culture techniques have been applied to HNSCC cells. In a recent study, Eke and colleagues (2011) used a laminin-rich extracellular matrix based 3D cell culture model to study the dual effects EGFR/FAK inhibition on cellular radiosensitivity of HNSCC cells.

Such techniques of tissue engineering are in addition, as with spheroids, limited in dimensions by gaseous and nutrient diffusion, however these are relevant for some studies.

### 1.8.4 Organotypic Culture

Such techniques aim to recombine cell types generated from a variety of cell lines, recreating the cell-cell and/or cell-matrix interactions. Organotypic cultures were first described for human skin cancer (Borchers et al., 1997, Boukamp et al., 1982) and have been since been used to study its invasion characteristics. Gaggiolo and colleagues (2007), for example, through collective imaging of invading co-cultures of carcinoma cells and stromal fibroblasts, revealed that carcinoma cells move within tracks in the extracellular matrix behind the leading fibroblasts. Organotypic cultures have been applied to different tumour types, such as ovarian, breast, prostate and oesophageal cancer, where they have provided important insights into, for example, the role of frequently altered genes in the biological behaviour and the mechanisms of tumour invasion (Okawa et al., 2007, Oyama et al., 2007). Such models however vary in their ability to mimic *in vivo* tissue

conditions and currently lack vasculature and normal transport of small molecules, host immune responses, and other cell-cell interactions.

## 1.9 Micro Fluidics

Having reviewed the vast array of techniques available to culture tumour cells *in vitro* and given an increased understanding of the microenvironment in tumour behaviour, the need for a better culture model becomes apparent. Although popular techniques such as spheroids and 3D matrixes attempt to recreate the three-dimensional *in vivo* characteristics and may incorporate differing cell types, the inadequate diffusion of gases and nutrients ultimately limit their application. The aim of these techniques is to recreate the tissue structure because, as described above, success with culturing the original tissue in an intact slice has largely been unsuccessful. The logical, and perhaps simplified next step, therefore, would be to modify organ culture in order to overcome the problems of diffusion, allowing pieces of tumour complete with all supporting cells and structures to be successfully cultured.

The unique properties of micro fluidic devices allow this to be done, providing a simple, reproducible and highly versatile biological tissue system. As previously described, cells exist *in vivo* in carefully maintained microenvironments within the three dimensional cell communities that form tissue. Within this microenvironment, cells are in close contact, receive a constant supply of nutrients, waste is removed, temperature is maintained and cell stress is minimal. For such homeostatic mechanisms to be maintained, efficient mass transport and mass exchange is imperative.

Micro fluidics relates to the "science and technology of systems that process or manipulates small ($10^{-9}$ to $10^{-18}$ litres) amounts of fluids, using channels with dimensions of tens to hundreds of micrometres" (Whitesides, 2006). The origins of micro fluidics lay in the microanalytical methods in capillary format (e.g. high pressure chromatography, capillary electrophoresis) first described by Jorgenson and Luckas (1981). Success in these methods prompted the development of more advanced and compacted formats for applications in microscale dimensions leading to the concept of the "miniaturized total chemical anaylsis system" or μ-TAS. The first applications of micro fluidic technologies were largely in the area of analytical

chemistry due to its advantageous properties relating to the use of small quantities of samples and reagents, the ability to carry out separations and detections with high resolution and sensitivity, low cost, short times for analysis and small footprints for the analytical devices (Manz, 1992).

Although early research in micro fluidics was initially dominated by studies within the disciplines of chemistry and physics at the microscale, the integration of cell biology with micro fluidics has recently become a major focus within the scientific community. Following the first cell culture within a microchannel (Doroszewski et al., 1977), the biological applications of micro fluidics have rapidly grown and now include areas such as cell and tissue culture, enzymatic analysis, DNA analysis (e.g., polymerase chain reaction and high-throughput sequencing), proteomics and clinical pathology.

### 1.9.1 Properties of Micro Fluidics

#### *1.9.1.1 Fluid Properties*

A fluid can be defined as a material that deforms continually under shear stress, or more specifically by the application of an external force attempting to displace part of the fluid elements at the surface layer (Ong et al., 2008). Fluid characterisations are further determined by its density, pressure and viscosity. The pressure of a liquid is determined by its depth, which within a micro fluidic device, should be constant. As the micro fluidic channels have inlets and outlets, any pressure difference induced externally at these openings is transmitted to the liquid, thereby inducing the liquid to flow. The fluid flow through microchannels is characterised by low Reynolds numbers (based on the relation between the magnitudes of the inertial and viscous forces). From empirical studies, Reynolds numbers larger than about 2300 correspond to turbulent flow; under this regime, inertial forces are dominant. When the Reynolds number is below about 2000, laminar (or creeping) flow predominates. Laminar flow allows multiple liquid streams to flow side by side without turbulent mixing, allowing a high degree of spatial control. Relatively low Reynolds numbers are attained at lower velocities, smaller dimensions, smaller densities, or higher viscosities. Therefore, in

microchannels, laminar flow regime is dominant due to the small dimensions, where the velocities of flow would have to exceed the speed of sound before the onset of turbulence (Walker et al., 2004). Whilst laminar flow reduces the shear damage to cells attributed to fluid motion, it also accounts for the favourable diffusion properties of micro fluidic devices described below.

This presence of laminar flow largely mimics the hydrodynamic fluid movement within the *in vivo* setting (Webster et al., 2010). In contrast, fluid movement within a culture flask is normally static, or turbulent if roller bottles are used to create fluid movement.

In addition, laminar flow in microchannels can allow for the partial treatment of single cells or tissue specimens. The simplest form of cell treatment in a microchannel is to secure a cell in a microchannel and flow the treatment over it, therefore exposing the whole cell to treatment. Increasing in complexity, a single cell can be subjected to two laminar fluid streams and therefore two different treatments. In the case of a spherical cell fixed within a microchannel, the interface between streams must be positioned exactly on the middle of the cell (Figure 1.21a). If the interface is not central, the cell will only be exposed to the contents of one stream (Figure 1.21b). The contents of stream B will diffuse toward the cell through stream A, but the amount which reaches the cell will depend on the flow rate and the diffusion coefficient of the species in stream B.

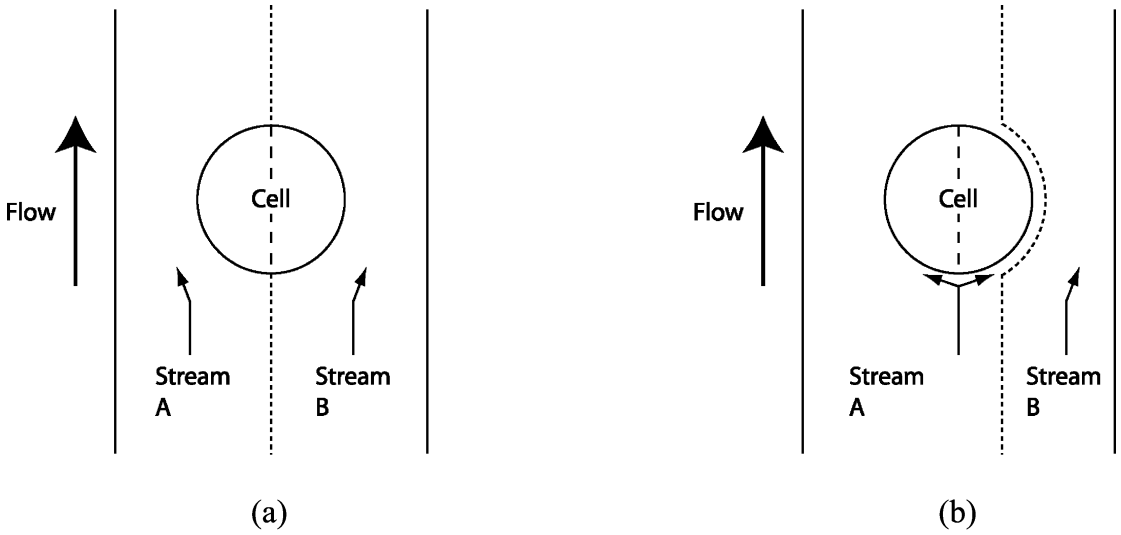



Figure 1.21 Schematic diagram demonstrating how laminar flow allows streams to flow side-by-side and mix only by diffusion. (a) Delivering a compound (e.g., stream B) to a cell using laminar flow can only be accomplished down the middle of the cell. (b) Otherwise, the cell will only be exposed to stream A and diffusion will be required to introduce elements from stream B to the cell. Adapted from Walker et al. (2004).

If further adapted for whole tissue culture, pieces of tissue, e.g. tumour biopsies could be subjected to several different treatment regimes at the same time, in order to determine the most effective regime, potentially revolutionising cancer treatment. In addition, more complex tissue surfaces will affect flow, and although laminar, will alter the distribution.

#### *1.9.1.2 Transport Properties*

Transport properties within micro fluidic devices can be divided into directed transport and statistical transport. Directed transport is controlled by exerting work on the fluid, resulting in volume flow of the fluid and is usually characterised by a direction and flow profile (Ong et al., 2008). This flow is often generated mechanically by a pump (i.e. pressure driven flow), or electrically by applying a voltage (electro-osmotic flow). Pressure driven flow allows fluid to be moved via positive or negative displacement and requires either a syringe pump mechanism (in which the whole fluid reservoir must be replaced when the fluid is exhausted) or a

peristaltic mechanism (where the reservoir is external to the pump, enabling the reservoir to be refilled without disrupting fluid flow). Electro-osmotic flow refers to the flow of fluid induced by the application of an electrical potential across a microchannel, causing ions in solution to migrate to the opposite charge lining the channel wall.

Conversely statistical transport is driven by entropy, where transport only occurs if, after transport, the fluid is more disordered than before. In the micro fluidic device this is seen as diffusion, whereby molecules will be transported from the side with a high concentration to the side with a lower concentration. This transport mechanism is readily seen *in vivo* for example in alveolar gaseous exchange of oxygen and carbon dioxide, glomerular filtration in the kidney and glucose uptake into cells. Likewise, the removal of waste products and delivery of nutrients is apparent within the micro fluidic system, where molecules can readily diffuse into and out of the constantly replenished growth media. This differs greatly from traditional cell and tissue culture models where cells are surrounded by their metabolic waste until the media is changed, e.g. every 2-3 days.

#### *1.9.1.3 Shear Effects*

Shear stress is felt by an object when a tangential force is applied to its surface. For a given maximum fluid velocity, the shear stresses present in a smaller microchannel are greater than those in a larger microchannel (Walker et al., 2004). This must be taken into consideration when designing microscale culture methods requiring fluid flow. When multiple cells are placed in a microchannel, intercellular spacing plays an important role in determining the fluid forces on each cell, with mathematical models available to predict the effects of changing cell spacing (Sugihara-Seki, 2000, Sugihara-Seki, 2001). From these models, the spacing between cells appears critical in determining whether or not the drag flow around cells is increased.

The maximal amount of shear that can be tolerated by the cells will depend on the cell type (if the device is being used for cell culture, with certain cells, e.g. endothelial) requiring shear stresses for adequate development (Nerem et al.,

1998). This property can be manipulated to stimulate *in vivo* growth conditions for example in osteoblastic cells (Leclerc et al., 2006) or to investigate disease processes, for example by mimicking cellular damage and disease progression (Gosgnach et al., 2000).

#### *1.9.1.4 Surface of Microchannels*

The surface properties of the microchannel are crucial in the functioning of the micro fluidic device and its ability to replicate the *in vivo* setting. As well as contributing to the flow properties within the channel via the surface energy of the channel walls, the high surface to volume ratios found within the micro fluidic channel help to mimic the *in vivo* setting. Cells are often grown in culture dishes with very low surface area to volume ratios (SAV); for example, a 35mm tissue culture dish with 1ml of medium in has a SAV of $11cm^{-1}$. Cells cultured in a microchannel of dimension 50μm x 50μm x 3cm (H x W x L) are in an environment with a SAV of $800cm^{-1}$ (Walker et al., 2004). This high ratio is advantageous, promoting efficient mass transport of gases via diffusion to and from cells, demonstrated *in vivo*, by the alveoli network of the human lungs providing a surface area for gaseous exchange of around $70m^2$ (Ganong, 1999). This also enables rapid temperature change and improves thermal transfer both by convective heat transfer at the solid/liquid interface and by rapid heat transfer in small fluid volume.

#### *1.9.1.5 Device Fabrication*

Early micro fluidic devices were based on silicon and glass substrates, manufactured by photolithography techniques and chemical etching. Glass based devices have more defined surface chemistry than plastics and better performance for separation purposes (Martin et al., 2006), can be easily sterilised, remain chemically robust and offer simple surface functionalisation and therefore are still widely used. They are however difficult to fabricate and relatively expensive to produce compared with poly(dimethylsiloxane) (PDMS) for example. Following the development of soft lithography techniques, plastics and elastomers have become popular substrates owing to the ease and speed of fabrication. The most commonly used material in soft lithography is PDMS, a silicon-based elastomeric material with

a number of desirable properties. It is cheap and easy to mould, non-toxic to cells, gas permeable, and has excellent optical properties, including low autofluorescence and optical transparency for imaging applications (Young and Beebe, 2010). While the many advantages of PDMS have established its popularity, some unfavourable characteristics have recently been revealed. The porous nature of PDMS can reduce its effectiveness when detection of trace elements is required and fluorescent dyes can have an increased propensity for surface binding (Kim et al., 2007). In addition, as cells are exposed to PDMS for longer durations, cell metabolism and proliferation are affected, possibly as a result of the presence of PDMS (Paguirigan and Beebe, 2009). Recent dissatisfaction with the use of PDMS has led to the consideration of other materials such as thermoplastics and biopolymers for micro fluidic use (Young and Beebe, 2010).

### 1.9.2 Biomedical Applications of Micro Fluidics

#### *1.9.2.1 Cell culture*

The applications of micro fluidic technology to cell culture are vast. A key area of development has included the isolation and enrichment of selected cell types from a heterogenous sample by a variety of means including deterministic lateral placement, shear stress and antibodies, dielectrophoretic field-flow separation and contactless dielectrophoretic separation ( Vykoukal et al., 2008, Green et al., 2009, Plouffe et al., 2009, Shafiee et al., 2010).

Successful cell growth has been achieved by many on glass and PDMS platforms, however recent studies have focused on the development of three dimensional constructs to better represent the ECM interaction, cell-cell interaction, soluble factors and mechanical forces seen *in vivo*. Various methods have been adopted to generate 3D environments including the use of scaffolds (Rowe et al., 2007, Ryu et al., 2007), bioreactors (Powers et al., 2002, Leclerc et al., 2006) and microstructured channels (Leclerc et al., 2006). Toh et al. (2007) developed a transparent 3D micro fluidic channel-based cell culture system that enabled optical monitoring of cells and cellular events. By facilitating the formation of cell-cell and cell-matrix interactions on the chip, carcinoma cells lines (HepG2, MCF7), primary

differentiated hepatocytes and bone marrow mesenchymal stem cells were maintained as perfusion cultures for up to a week, after which their 3D cytoarchitecture, cell-specific functions and differentiation competence were well preserved. These studies however were all based on the culture of homogenous cell lines, with the differing cells of the true tumour microenvironment not represented.

#### *1.9.2.2 Clinical Diagnostics*

Micro fluidic devices are well suited to the handling of clinical samples, benefiting from rapid throughput, small sample size and accuracy and the availability to provide low-cost, single-use devices. The use of micro fluidic devices as biosensors to detect specific cellular biochemical outputs mediated by isolated enzymes, organelles, whole cells, tissues, or immunosystems is in widespread use. A multitude of commercial micro fluidic based biosensors have been developed for measuring various body compounds such as blood gas (Lam and Atkinson, 2007, Huang, 2007), glucose (Huang, 2007) and cholesterol (Aravamudhan et al., 2007).

One of the advantages of micro fluidic technology is the ability to combine multiple functions. Mohammed et al. (2009) for example, describe a micro fluidic device to perfuse pancreatic islets whilst simultaneously characterising mitochondrial membrane potential and intracellular calcium, as well as off-chip quantification of secreted insulin by enzyme-linked immunosorbent assay (ELISA), offering a means for the rapid assessment of tissue quality immediately following donor isolation. This is however yet to be adopted into clinical practice as the *in vitro* functional assay results have yet to be correlated with *in vivo* islet function.

In this context, the integration of electrochemical detection of genetic and protein cancer markers with micro fluidic technology has been of interest. The approach consists of the immobilisation of a multitude of marker probes on an electrode array, each electrode being functionalised with a particular probe consisting of antibodies or antigens in the case of immunosensors or a short oligonucleotide sequence in the case of genosensors. Henry et al. (2009) developed a packaged micro fluidic cell for the multiplexed detection of tumour markers such as carcinoembryonic antigen (CEA) and prostate-specific antigen as well as DNA

breast cancer markers such as oestrogen receptor-α, plasminogen activator urokinase receptor, EGFR and erythroblastic leukemia viral oncogene homolog 2. They demonstrated that several parameters including both proteins and genes could be detected on a single chip with reproducibility (relative standard deviation <8% for CEA and PSA detection) and high sensitivity.

Micro fluidic based analysis has been applied to automated cell quantification for circulating tumour cell (CTC) detection (Nagrath et al., 2007), cell detection in biosensors (Oh et al., 2007) and CD4 counting devices (Alyassin et al., 2009). Alyassin et al. (2009) describe an automated micro fluidic device for high throughput quantification of florescent cell image analysis using HIV-infected whole blood samples. Compared with manual counting, the automated device performed similarly in terms of speed and accuracy with small cell numbers, however the micro fluidic device was more accurate and >100 times faster for multiple-colour stained cells, or when large numbers of cells (i.e. >500) were present. Lee et al. (2009) quote a high sensitivity and detection rate (comparable with conventional methods of flow cytometry and Western blot analysis) for the measurement of cancer cells in tumour aspirate. Using a diagnostic magnetic resonance sensor, as few as 2 cancer cells in 1μl of tumour aspirates were detected, with expression profiling in under 15 min. Nagrath et al. (2007) describe a microchip to identify CTCs in the peripheral blood of patients with metastatic lung, prostate, pancreatic, breast and colon cancer. The CTC chip identified circulating tumour cells in 115 of 116 (99%) samples, with a range of 5 - 1281 CTCs per ml and approximately 50% purity. In addition, CTCs were isolated in 7/7 patients with early-stage prostate cancer.

#### *1.9.2.3 Point of Care Testing*

This ability to detect a variety of biological compounds, coupled with the potential for automation and integration on to a portable device, means that micro fluidic devices are well-suited to point-of-care diagnostic instrumentation (point of care testing, POCT). POCT is defined specifically as “testing at or near the site of patient care” (Kost, 2002). Historically, all diagnostic testing was performed at the patient’s bedside, however modern medicine has dictated the migration of diagnostic testing to the central laboratory due to size and expense of analytical

equipment and operator dependence. One rationale for POCT is that it may streamline healthcare and improve clinical outcomes (Price and Kricka, 2007). For example, the finding of a high blood glucose level in a diabetic patient can be acted upon immediately by administration of the appropriate therapy. Weigl et al. (2008) suggest that POCT is of use in developing-country health care, home testing in developed countries, and diagnostic and bioanalytical disposables for use in natural or man-made bioemergencies by first responders.

The salient features of a point of care device include the following: short time to results, ease of use (includes sample preparation and fully automated sample processing), portability, small sample volumes (< 30 µL requirement allows finger stick rather than venous sample), multifunctionality (capable of combining different types of tests required along the path of care) and low cost (Sista et al., 2008). Other important requirements include: self-contained reagents, self-calibration and connectivity to laboratory information systems.

Lateral flow tests were among the first successfully commercialised micro fluidic products in POCT. Using the capillary test strip platform, a variety of diagnostic application are possible including the detection of health biomarkers in pregnancy (Chard, 1992), heart attack (Clark et al., 2002), blood glucose (Bohme et al., 2003) and metabolic disorders (Lou et al., 1993), the detection of small molecules, e.g. mycotoxins in food (Krska and Molinelli, 2009), antibiotics (Xie et al., 2009) and detection of infectious agents such as anthrax (Carter and Cary, 2007), salmonella (Ho et al., 2008) and viruses (Edwards and Baeumner, 2009).

The application of micro fluidic-based POCT to immunoassays is of interest. The conventional ELISA approach has a number of limitations for its use in resource-limited settings, because it requires relatively long assay times (4 – 6 hours), cumbersome liquid handling, and large amounts of expensive reagents and equipment. Several rapid diagnostic tests have been developed for detection of various entities using immunoassays. Herr et al. (2007) developed a clinical POC diagnostic that enabled rapid quantification of a putative biomarker of periodontal disease in saliva by using a monolithic disposable cartridge designed to operate in a

compact analytical instrument. This device integrated sample pretreatment (filtering, enrichment, mixing) with electrophoretic immunoassays to measure analyte concentrations in minimally pretreated saliva samples. Using 20μl of saliva, they demonstrated rapid (<10 min) measurement of the collagen-cleaving enzyme matrix metalloproteinase-8 (MMP-8) in saliva from healthy and periodontally diseased subjects. By validation with conventional measurements of salivary MMP-8, the authors concluded that their device provided rapid, reliable measurement of proteinaceous disease biomarkers in biological fluids. This study was however more of a 'proof of concept' as additional investigations regarding MMP-8 as a biomarker for periodontal disease and its ability to predict disease progression, as well as a means to monitor therapeutic efficacy are needed.

#### *1.9.2.4 Pharmacological Studies*

Drug discovery is often impeded by the poor predictability of *in vitro* assays for drug toxicity. Micro fluidic devices are suited to toxicological studies and drug therapy studies given their potential for the faster assay times and ability to handle small samples. They also pose significant advantages over traditional *in vitro* models, allowing human tissue samples, more closely resembling the *in vivo* state, to be tested, bridging the gap between pre-clinical and clinical drug testing.

Drug development is a costly and time consuming, with approximately only one in ten drugs entering clinical trials finally being approved (Kola and Landis, 2004), one of the main causes for such a high attrition rate being unforeseen lack of efficacy or toxicity. A major disadvantage of conventional multi-well plate assays is the lack of multi-organ interactions, meaning that the pharmacokinetics of drugs, which plays a significant role in determining their pharmacological effect, cannot be assessed.

Micro fluidic devices with perfusion cell culture offer a great potential for drug screening in a high-throughput manner and can be useful for reproducing the pharmacokinetics of drugs. Multiple components such as a metabolizing entity (e.g. liver) and a target structure (e.g. tumour) can be connected with fluidic channels for multi-organ interactions. Ma et al. (2009) developed a three-layer micro fluidic

system to test metabolism-dependent toxicity of drugs, consisting of a top-layer for feeding drugs, a middle layer with human liver microsomes, and a bottom-layer for cell culture chambers. By studying the metabolism of UDP-glucuronosyltransferase, the drug-drug interactions between acetaminophen and phenytoin and their effect on hepG2 cells were studied, with an increased hepG2 cytotoxicity seen in response to drug treatment. Sung and Shuler (2009) developed a microscale cell culture analogue (μCCA), also known as 'body-on-a-chip', to assess the effects of 5FU. Three separate cell types, namely liver (hepatoma calls HepG2/C3A), tumour (colon cancer, HCT-116) and bone marrow (myeloblasts, Kasumi-1) were cultured in separate chambers on a single chip, interconnected by channels mimicking the blood flow pattern. The device was able to reproduce the metabolism of the oral pro-drug tegafur in the liver, reproducibly initiating cell death by 5FU, while 96-well plate cultures were unable to demonstrate significant cell metabolism.

Although the examples above used micro fluidic technology to integrate different populations of cells rather than culture whole tissue, its success provides proof of concept of the potential of micro fluidics to recreate *in vivo* physiological systems. More recently, Midwoud et al. (2010b) have designed a micro fluidic system for the perfusion of precision-cut intestinal slices, and for the sequential perfusion of intestinal and liver slices mimicking the *in vivo* first pass situation. By sequentially perfusing rat intestinal and liver tissue slices in a two-compartment co-culture perfusion system with a continuous flow of medium, the authors directed metabolites or other excreted compounds formed by an intestinal slice to the second compartment containing a liver slice. The interplay between these two organs was demonstrated by exposing the slices to the primary bile acid, chenodeoxycholic acid (CDCA). CDCA induced the expression of fibroblast growth factor 15 in the intestinal slice, which resulted in a stronger down-regulation of the enzyme, cytochrome P450 7A1 in the liver slice in the second compartment than when the liver slice was exposed to CDCA in a single-microchamber biochip.

Micro fluidic devices have also been used to integrate the study of drug pharmacokinetics (PK) and pharmacodynamics (PD) in order to predict the time-course of pharmacological effects from a dosage. Further to the above study, Sung

et al. (2010), by combining a mathematical modelling approach (PK–PD modelling) with their *in vitro* experimental approach, demonstrated that each cell type exhibited differential responses to 5FU, and the responses in the micro fluidic environment were different from those in static environment, clearly supporting the need for 'tissue' based modelling.

Micro fluidic technology has been particularly useful in the analysis of chemotherapy resistance cell lines, particularly in the study of lung cancer. By combining immunofluorescence and chip electrophoresis, glucose-regulated protein 78 was found to correlate with resistance to topoisomerase II inhibitor-VP-16 in human lung cancer cell lines (Ying-Yan et al., 2008). Similarly, assay of the expression of multidrug resistance related protein P-gp and the correlation between the expression of P-gp and resistance to VP-16 was reported by Zhao et al. (2010). The results indicated that the cells could grow and spread well for 4 days in the micro fluidic system. Immunofluorescence assay of P-gp showed weak fluorescence for the verapamil-pretreated cells compared with that of non-pretreated cells and apoptosis analysis demonstrated a twofold increase in the percentage of apoptotic cells in the verapamil-pretreated group compared with that of non verapamil pretreated. These results indicate that P-gp plays an important role in the resistance to VP-16 in a human lung cancer cell line and highlight the role of micro fluidic cell culture in determining mechanisms of drug resistance.

#### *1.9.2.5 Tumour Spheroids in Micro Fluidic Chambers*

As described in section 1.8.3, spheroids, a commonly used tumour model go some way to recreating the complex multicellular tissue structure. Their use is however limited, as previously outlined, although can be enhanced when integrated with micro fluidic technology. Multicellular spheroids have been cultured within a micro fluidic device, as first described by Torisawa et al. (2007). By introducing cells in suspension through silicon microholes into microwells, cellular spheroids were formed. By demonstrating the continued production of albumin from the hepatoma cell line (HepG2), the spheroids functioned within the device for 2 weeks. Similarly, Hsiao et al. (2009) used a two-layer micro fluidic system to culture 3D multicell type

spheroids of fluorescently labelled metastatic prostate cancer cells (PC-3 cell line), osteoblasts and endothelial cells. Culturing the cells in this environment greatly decreased the proliferation rate of PC-3 cells without reducing viability, suggesting this method may more faithfully recapitulate the *in vivo* growth behaviour of malignant cancer cells within the bone metastatic prostate cancer microenvironment.

The ability to manipulate the flow characteristics within the micro fluidic device has been utilised by Hu and Li (2007) to study the growth dynamics of avascular tumours. A three-dimensional flow and nutrient transport model, incorporating the MTS growth model was used to study the influence of nutrients (oxygen and glucose) supply and distribution on the MTS growth. Numerical simulations based on the EMT6/Ro tumour cells showed that the continuous-flow perfusion was a more efficient method of delivering nutrients to the MTS than the diffusion-only static culture. Additionally, the flow shear stress exerting on the MTS within the microchannel bioreactor was estimated to be far below the critical value to affect growth, allowing for an increase in perfusion velocity to satisfy the higher nutrient requirement by the growing tumour spheroids. Rosano et al. (2009) developed a synthetic microvascular network on a PDMS chip that attempted to recreate the bifurcations, tortuosities, and cross-sectional changes found in microvascular networks *in vivo*. By mapping microvascular networks from a cremaster muscle using a modified Geographical Information System, the networks were successfully cultured with bovine aortic endothelial cells, which reached confluency 3-4 days after seeding.

Micro fluidic devices have also been used to study the effects of chemotherapy agents when the *in vitro* conditions have been manipulated to mimic the microenvironment gradients present in tumours. Walsh et al. (2009) formed tumour spheroids (from colonic cancer cells) within micron-scale chambers exposed to medium perfusion on one side to create linear nutrient gradients. Fluorescence microscopy was used to demonstrate that the cell mass contained viable, apoptotic, and acidic regions similar to *in vivo* tumours. The device was also able to characterize the transport differences between a passively diffusing therapeutic

(doxorubicin) and actively penetrating vectors (Salmonella bacteria) aiding the understanding of diffusion resistance within solid tumours.

#### *1.9.2.6 Whole Tissue Culture*

Whole tissue culture is perhaps the most exciting and novel application of micro fluidics. This is the area most in its infancy, but given the limitations of current culture techniques, has the ability to revolutionise the way we grow tumour tissue. Culturing whole tissue in a micro fluidic device can offer the recreation of the tumour microenvironment, maintenance of complex cellular interactions whilst benefiting from the technological applications of micro fluidics. To date, only 7 papers (see Table 1.6) have been published on whole tissue culture and of these, only 1 (Webster et al., 2010) has focused on the utilisation of micro fluidic devices for the maintenance of whole pieces of tumour.

Table 1.7 Published literature on the use of micro fluidics for whole tissue culture, to January 2011.

| *Tissue* | *Application* | *Reference* |
|---|---|---|
| Rat hippocampus | 600μm slices perfused in microfabricated glass slides for 5hr as demonstrated by eptileptiform activity | Passeraub et al. (2003) |
| Rat medulla | 700μm slices perfused on PDMS slices for 3 hr with evidence of respiratory-related motor output. Solutions controlled to target specific areas demonstrating evidence of laminar flow. | Blake et al. (2007) |
| Rat liver | 4 $mm^3$ slices perfused in glass micro fluidic devices for up to 70 h. Viability studies by Lavacell™ staining, pico green assay and LDH release. Functionality by albumin and urea synthesis | Hattersley et al. (2008) |
| Mouse brain cortex | 700μm slices perfused on PDMS micro fluidic devices. Viability up to 5 days demonstrated by fluorescent nuclear staining and on histoarchitectural examination | Rambani et al. (2009) |
| Rat liver | 100μm in PDMS micro fluidic devices for up to 24 hr as measured by viability of 7-ethoxycoumarin and LDH release | Van Midwoud et al. (2010a) |

| | | |
|---|---|---|
| Rat/human heart | 4mm$^3$ slices maintained for 3.5 – 5 hr in PDMS micro fluidic devices. Viability analysis by effluent levels of LDH and hydrogen peroxide in combination with *in situ* electrochemical measurement of the release of reactive oxygen species. | Cheah et al. (2010) |
| Human colorectal tumour | 2mm$^3$ slices maintained for over 3 days in a glass micro device. Functionality demonstrated by changes in release of vascular endothelial growth factor in response to hypoxia. | Webster et al. (2010) |

The majority of reports on the use of micro fluidic devices for culturing whole pieces of tissue have centred upon studies using brain tissue. The maintenance of 'thick' slices of brain was first described by Passeraub et al. (2003). They used fabricated glass with arrays of micropillars to replace the nylon mesh used in classical interface chambers in order to confine the flow and uphold hippocampal slices (600μm thick) at the interface between perfusate and oxygen. Using a zero-$Mg^{2+}$ model of epileptiform activity, spontaneous single and multi-spike bursts in the CA3 region of a rat hippocampal brain slice were observed for more than 5 h. Blake et al. (2007) used a three-layer PDMS micro fluidic device to culture medullary slices (approximately 700μm thick). Their fabrication technique included rapid production of PDMS layers (using a PDMS master allowed for a 2 h microfabrication time rather than 10 h if a negative photoresist master was used) that could be applied to brain slices of different shapes and sizes. The slices in this chamber spontaneously produced rhythmic, respiratory-related motor output for up to 3 h. They also demonstrated how the laminar flow exhibited by the micro fluidic chamber allowed controlled solutions to target specific areas of the brain slice based on the input flow rates. A stream of $Na^{+}$-free solution was focused on one half of a medullary slice to abolish spontaneous neural activity in that part of the brain slice, whilst the other half remained active. By modifying their custom-made microperfusion chamber to facilitate perfusion of oxygenated nutrient medium throughout the tissue thickness with concomitant removal of depleted medium and catabolites, Rambani et al. (2009) enhanced the viability of the thick organotypic brain slice cultures. By investigating a range of flow rates, they demonstrated up to 84.6% viability after 5 days in their 700μm thick organotypic brain slices, compared with the unperfused control cultures. Functional activity was confirmed by demonstrating continued spontaneous electrical activity and preservation of *in vivo* cyto-architecture was studied using microscopy.

The maintenance of liver tissue biopsies has also been described (Hattersley et al., 2008, van Midwoud et al., 2010a). Using a glass microchip, Hattersley et al. (2008) maintained pieces of liver tissue ($4mm^3$) in a viable state for over 70 h. Functionality was demonstrated by continued production of albumin and urea. This

was corroborated by Lavacell$^{TM}$ staining of viable cells as well as final disaggregation on-chip using a collagenase digestion to show cell viability by Trypan blue assay. Based on a PDMS device, van Midwoud et al (2010a) maintained precision-cut liver slices (of 100μm thickness) within a micro fluidic perfusion system. Liver slices were viable for at least 24 h with the compound, 7-ethoxycoumarin (7-EC) used to demonstrate the preservation of cell metabolism.

Additionally, a micro fluidic device has been developed to maintain viable heart tissue samples (Cheah et al., 2010). This device allows rat or human heart tissue to be studied under pseudo *in vivo* conditions. By using effluent levels of lactate dehydrogenase (LDH) and hydrogen peroxide as markers of damaged tissue in combination with *in situ* electrochemical measurement of the release of reactive oxygen species, heart tissue (2mm$^3$ thick) was maintained for up to 5 h.

Most recently, our research group published a communication relating to the culture of human colorectal tumour in a micro fluidic device (Webster et al., 2010). Using a similar glass micro fluidic device to the one used in this study but employing a peristaltic rather than a syringe-pump driven perfusion system, the response of 2mm$^3$ tumour biopsies to hypoxic conditions was measured by expression of VEGF by off-chip ELISA. Tumour tissue showed a greater increase of VEGF compared with that of normal tissue non-neoplastic colorectal tissue after a period of hypoxia ($N_2$/$CO_2$ gassed media). This demonstrated both biopsy viability within the device and the potential use of VEGF as a biomarker for monitoring response of tumour biopsies to changes in the microenvironment.

Whole tissue perfusion represents the ultimate in preservation of the tissue microenvironment. The studies listed in table 1.7 and highlighted above demonstrate viability and functionality of whole tissue within micro fluidic devices. Furthermore, the potential for pharmacology studies and application to tumour biology is being realised.

## 1.10 Study Aims

As the biological application of micro fluidic technology is a relatively novel concept, particularly with regards to whole tissue culture, the first aim of this study was to determine the stability of HNSCC biopsies within a micro fluidic device, using markers of cell death and proliferation. Lactate dehydrogenase (LDH) and mitochondrial metabolism of water soluble tetrazolium (WST-1) were assayed off-chip to determine cell death and proliferation respectively. These biochemical markers were coupled with subjective assessment of histo-architectural changes following incubation within the micro fluidic device.

The second aim was to optimise the platform for studying chemotherapy administration in HNSCC. This consisted of determining the most suitable doses of chemotherapy agents to use on HNSCC within the micro fluidic system and assessing tumour cell death in response to the addition of various chemotherapy agents, as measured by LDH release.

Finally the thesis work evaluates the potential of the micro fluidic approach for widespread clinical applicability, highlighting the key advantages, as well as the hurdles, which remain to be overcome prior to implementation.

# 2 Materials and Methods

All chemicals and reagents were supplied from Sigma-Aldrich Ltd, Dorset, UK and were of tissue culture or molecular grade as appropriate unless otherwise attributed.

## 2.1 Obtaining Tissue for Use in Micro fluidic Devices

### 2.1.1 Ethical Approval

Ethical approval was obtained from the Hull and East Yorkshire Ethics Committee for the use of excess tissue from fresh surgical resections undertaken as part of normal patient treatment for the study 'Development of a prototype micro fluidic device for the study of cell functions within a tissue environment' (LREC 07/H1304/105). This was also approved by the Hull and East Yorkshire NHS Trust Research and Development Department (R0568). Any excess tissue could be used, however no patient details were stored in association with the tissue; only the tissue's histological details, i.e. site and stage, were recorded. During the project, ethical approval was then sought specifically for 'The use of micro fluidic devices for the evaluation of tumour biology and treatment response in head and neck cancer biopsies and correlations with patient outcome' (LREC 10/H1304/7; NHS Trust R&D R0897). This enabled the recording of patient demographics and treatment plans to be recorded allowing correlation between the *in vitro* findings and clinical management.

### 2.1.2 Patient Selection

Patients with known HNSCC, undergoing a primary resection with curative intent of either their primary tumour or metastatic lymph nodes (with a prior diagnosis of HNSCC at diagnostic biopsy) on the Ear, Nose and Throat ward at Castle Hill Hospital, Hull and East Yorkshire NHS Trust were approached. For the tumours used in sections 3.1, 3.2 and 3.3, the tissue was used anonymously. For tumour studies in section 3.4 informed consent was obtained prior to theatre and an information pack given (Appendix II).

Although the patients were largely selected due to the timing of their presentation for surgery, some patients (in section 3.4 only) were excluded due to histopathological discrepancies. These were:

1) Ambiguous primary sites meaning that the principle site could not be definitely identified

2) Unknown primary site

3) Non-squamous cell carcinoma or mixed tumour histopathology and/or

4) A history of previous chemo or radiotherapy prior to the date of resection.

### 2.1.3 Tissue Sampling

Once the tumour specimen had been removed, a biopsy measuring approximately 1-5 $cm^3$ (depending on availability) was dissected by the Consultant head and neck surgeon (Professor N Stafford or Mr J Jose) under aseptic conditions. Specimens were dissected from either or both the primary tumour and involved lymph nodes, depending on the regional spread. At no time were specimens dissected if it was felt that pathological staging would be affected. The head and neck Consultant Histopathologist (Dr L Karsai) was advised of the removal of part of the tumour for research purposes.

The biopsy was then placed into a sterile tube containing 20 ml of Dulbecco's modified Eagle's medium, (DMEM, PAA Laboratories, Yeovil, Somerset, UK) supplemented with 10% (v/v) heat inactivated filtered (0.22 μm filter, Millipore™, Watford, UK) bovine serum, 3% (w/v) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer), 1% (w/v) penicillin/streptomycin, 1% (w/v) non-essential amino acids (PAA) and 1% (w/v) glutamine and immediately transferred to the laboratory. If fresh pieces of tumour were used in micro fluidic devices, the biopsy was immediately prepared and placed in the primed devices as described below. For frozen tumour studies or for any excess tumour from fresh studies, the tissue was divided into $1cm^3$ pieces under aseptic conditions, wrapped in silver foil, snap frozen in liquid nitrogen (-196°C) within 15 minutes of tumour dissection in theatre, then stored at -80°C until required.

## 2.2 Device Fabrication

All of the glass micro devices used in this study were manufactured by Dr. S Clark in the Department of Chemistry at the University of Hull, using a photolithographic technique in accordance with published literature (McCreedy, 2001). Photolithography refers to the selective removal of parts of a thin film or the bulk of a substrate, using light to transfer a geometric pattern from a photo mask to a light-sensitive chemical photo resist, or simply "resist", on the substrate.

To draw the required channel structure, AutoCAD LT software computer assisted design (CAD) software package was used. The geometric channel pattern outline was then transferred to a mask or thin film (the photo mask), leaving a blank geometric pattern on a black background. This allows light to penetrate through the mask in order to expose the channel design on the glass wafers (Telic, Valencia, US).

Glass wafers of 1 mm thickness coated with the chromium layer and a photoresist layer were used as illustrated in Figure 2.1. Exposure was achieved by placing the mask on a ultra-violet (UV) exposure unit with glass wafers placed on top of the channel design, in a process known as 'contact printing', commonly used for small volume production. The exposed glass wafer was then immersed in developer solution (Chestech Ltd., Rugby, Warickshire, UK) for 60 s, rinsed under running water and then dried by nitrogen ($N_2$) exposure. Following this, the glass wafer was immersed in Chrome Etch MS8 solution (Chestech) for 60 s, or until the pattern was fully exposed on the glass. The plate was then rinsed with deionised water and blown dry with $N_2$ gas.

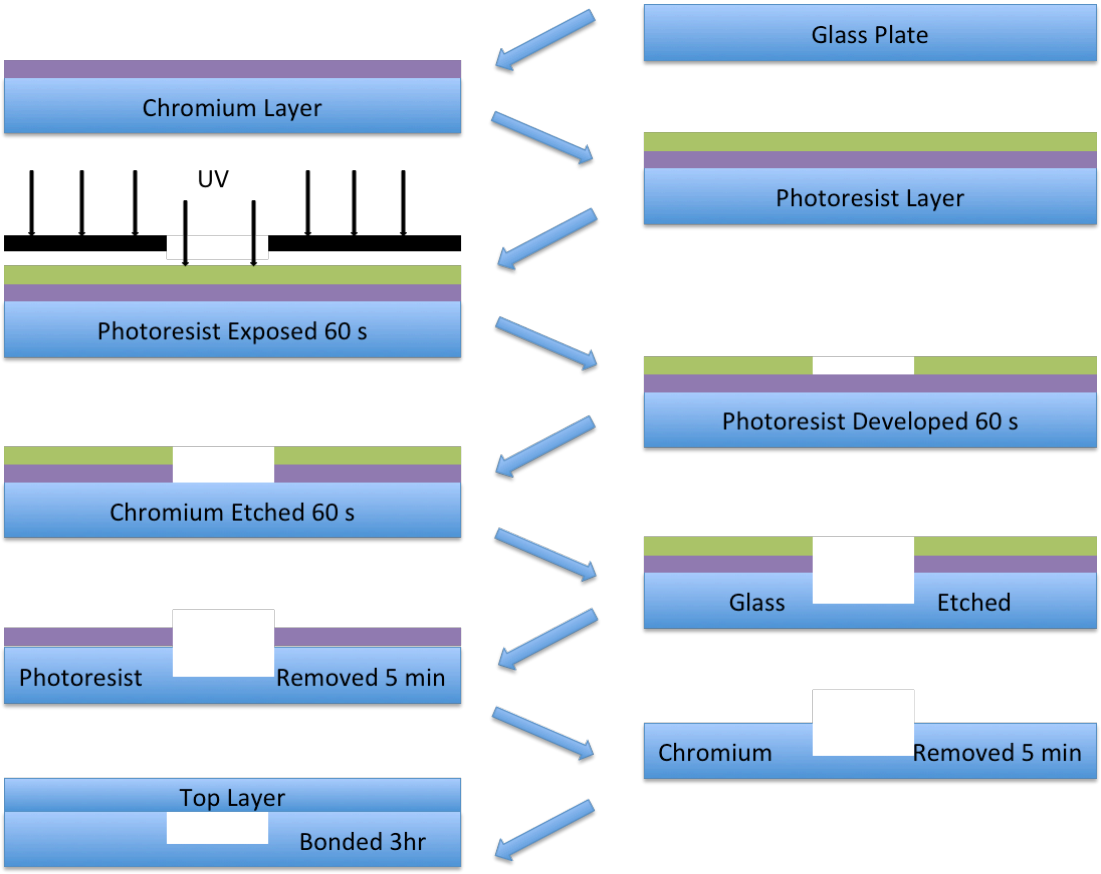



Figure 2.1 Diagram of the photolithography and wet etching method of micro fluidic chip fabrication

The wet etching process then followed using hydrofluoric acid (48 % v/v), ammonium fluoride (40 % v/v), hydrochloric acid (38 % v/v) and deionised water. Etching time was dependant on the depth of the channel required. For this study, the channel dimensions were of 190 µm width and 70 µm depth (Figure 2.2). The depth was subsequently verified using light microscopy. Due to the nature of the etching process, etching occurs both widthways as well as down-wards, creating channels with a trapezoidal shaped cross section.

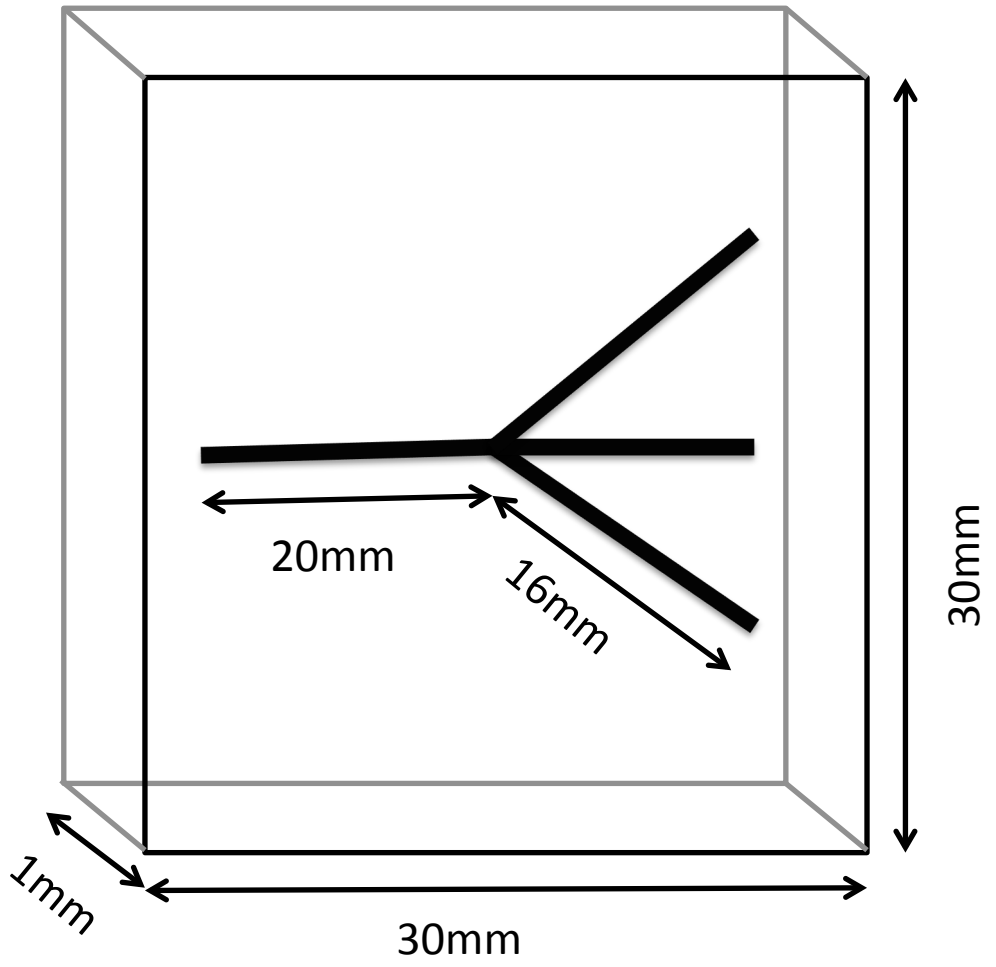



Figure 2.2 Schematic diagram of the etched base glass plate with a channel width of 190 µm and depth of 70 µm.

The glass beneath the photoresist and chrome layers was etched whilst the top two layers remained intact. The photoresist layer was removed from the etched plates by immersion in acetone for several minutes until all of the photoresist was removed. The glass plates were then rinsed with water and dried with $N_2$ gas. To remove the chrome plate, the plates were placed in the chromium etchant, Chrome Etch MS8, for several minutes then removed and allowed to dry. At this stage, access holes were drilled in the 3 mm thick top plate, using 1.5 mm drill bits by standard drilling techniques as shown in Figure 2.3.

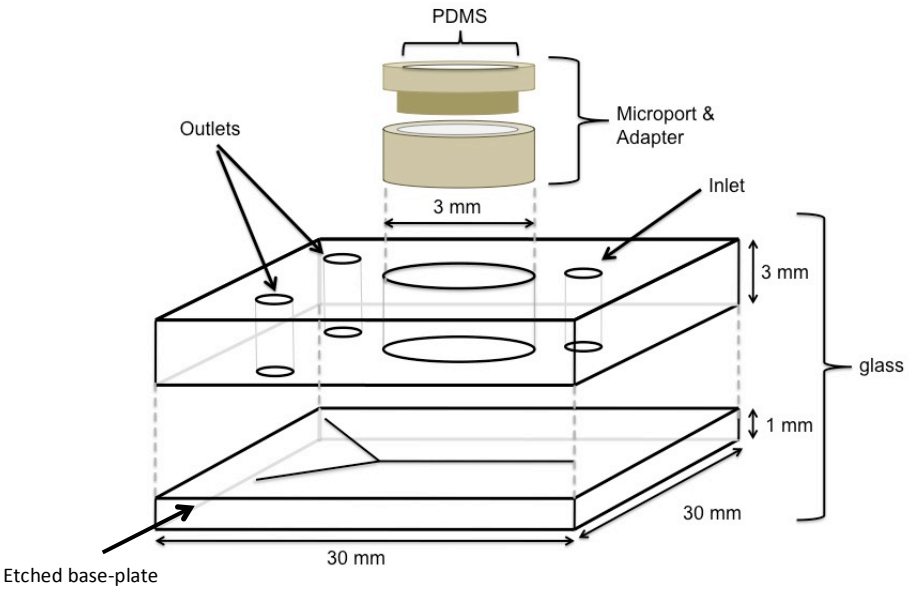



Figure 2.3 Schematic representation of the micro fluidic device used (Courtesy of S. Hattersley)

A central hole with a diameter of 3mm was drilled into the top plate to provide a chamber for the tissue. The tissue chamber is cylindrical with an internal volume of 21 μl. The top plate with the drilled holes was then thermally bonded onto the etched bottom plate in a furnace at 590 °C for 3 h.

A microport fitting (Anachem Ltd, Luton, Bedfordshire, UK) was finally bonded to the surface of the top 3 mm glass layer (described in section 2.3.2) so that the circular tissue cavity could be sealed by way of an English threaded adapter (Anachem). The adapter was filled with PDMS (Dow Corning Ltd, Barry, Vale of Glamorgen, UK) to allow gaseous exchange to occur (Ng et al., 2002) as shown in Figure 2.4. The PDMS was made in accordance with manufacturer's instructions (Dow-Corning and Information, 2005); mixing 1 part curing agent to 10 parts base. The bung was filled with PDMS and allowed to cure for 48 h at room temperature before use.

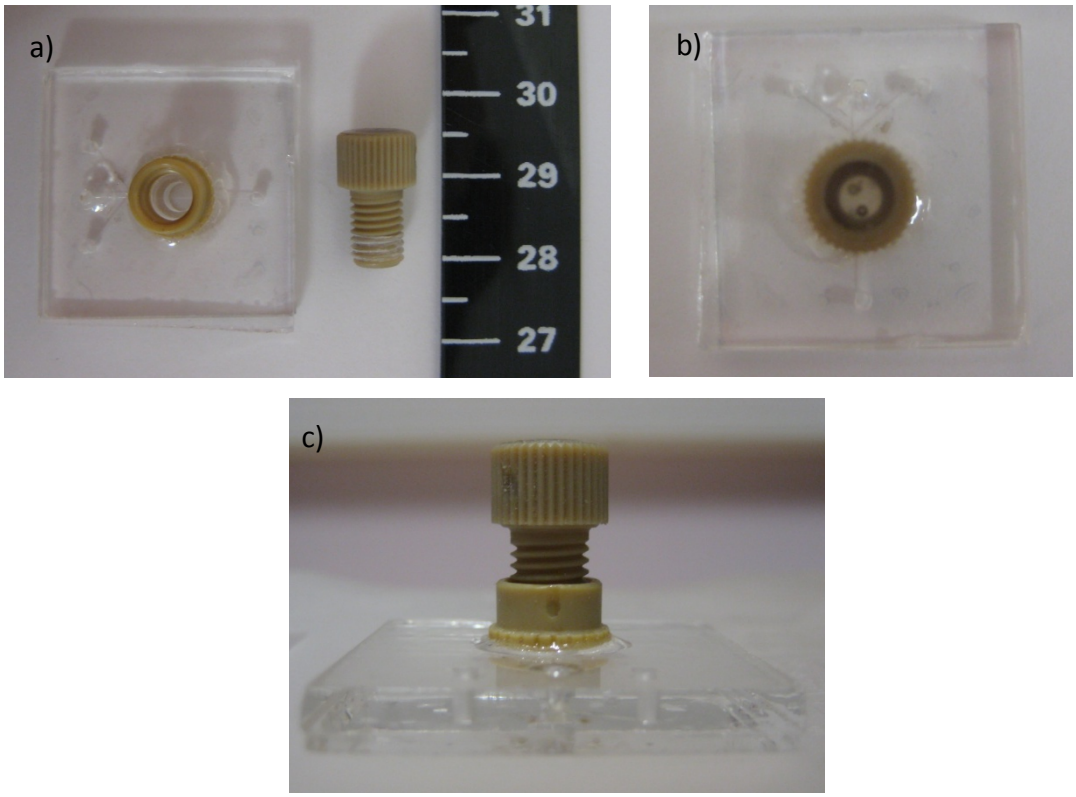



Figure 2.4 Images of the micro fluidic devices a) with the threaded adapter along side, b) top view of the PDMS threaded adaptor screwed into the microport fitting and c) side view of b).

## 2.3 The Micro fluidic System

### 2.3.1 System Components

The micro fluidic system used during the study comprised of the glass device (with microport and PDMS bung), Teflon® tubing, incubator and hydrodynamic pumps.

A Harvard PhD 2000 syringe pump (Harvard Apparatus Ltd, Kent, UK) was connected to the device *via* 0.8 mm ID x 1.58 OD TFE Teflon® (Sigma). The length of the tubing from the 20 ml syringe (BD™, UK) to the device was 30 cm and outlet tubing was 8 cm long as shown in Figure 2.5. A 0.22 μm filter (Millipore™) was fitted between the syringe and the tubing to reduce the possibility of bacterial and particulate contamination as well as minimising the occurrence of bubbles into the device. To connect the tubing to the filter, a 3-part adaptor (Upchurch Scientific,

WA, US) was used. For overnight effluent collection, a 1.5 ml polypropylene centrifuge tube, with a hole in the lid covered with parafilm to stop contamination and leakage, was placed on the end of each outlet tubing to collect effluent. For 2 hourly sample collections, a 0.5 ml tube was used (Figure 2.5b). The micro fluidic devices attached to outlet tubing and collection tubes plus the majority of the inlet tubing was placed in warm air box, maintained at 37°C (Figure 2.5a).

### 2.3.2 Device Assembly and Priming

Before use, the glass microchips, tubing, 3-part adaptor and microport adaptor were autoclaved at 121°C for 20 minutes. Once sterilised, the microport adaptor was bonded to the glass chip using Araldite® Precision Adhesive (Huntsman Advanced Materials, Basel, Switzerland) and allowed to set at 60°C for 4 hours. Similarly, the cut end of a 200 μl pipette tip was bonded to one end of the inlet and outlet tubing lengths. Once assembled as shown in Fig. 2.5, the system was primed with 70% (v/v) ethanol/water, pumped through the device for 15 minutes at 10 μl $min^{-1}$ followed by a rinse with autoclaved, distilled water for 15 minutes at 10 μl $min^{-1}$. Following this, the standard supplemented media as described in section 2.1.3 was pumped through the system for a minimum of 30 minutes at 5μl $min^{-1}$.

The tumour, either fresh or if frozen, allowed to defrost for 2 minutes, was cut into approximately 4 $mm^3$ slices and individually weighed in aseptic conditions. The weight of each slice was recorded with a weight of approximately 5-10 mg being aimed for. The tumour was then placed in the central well of the glass micro fluidic device under aseptic conditions.

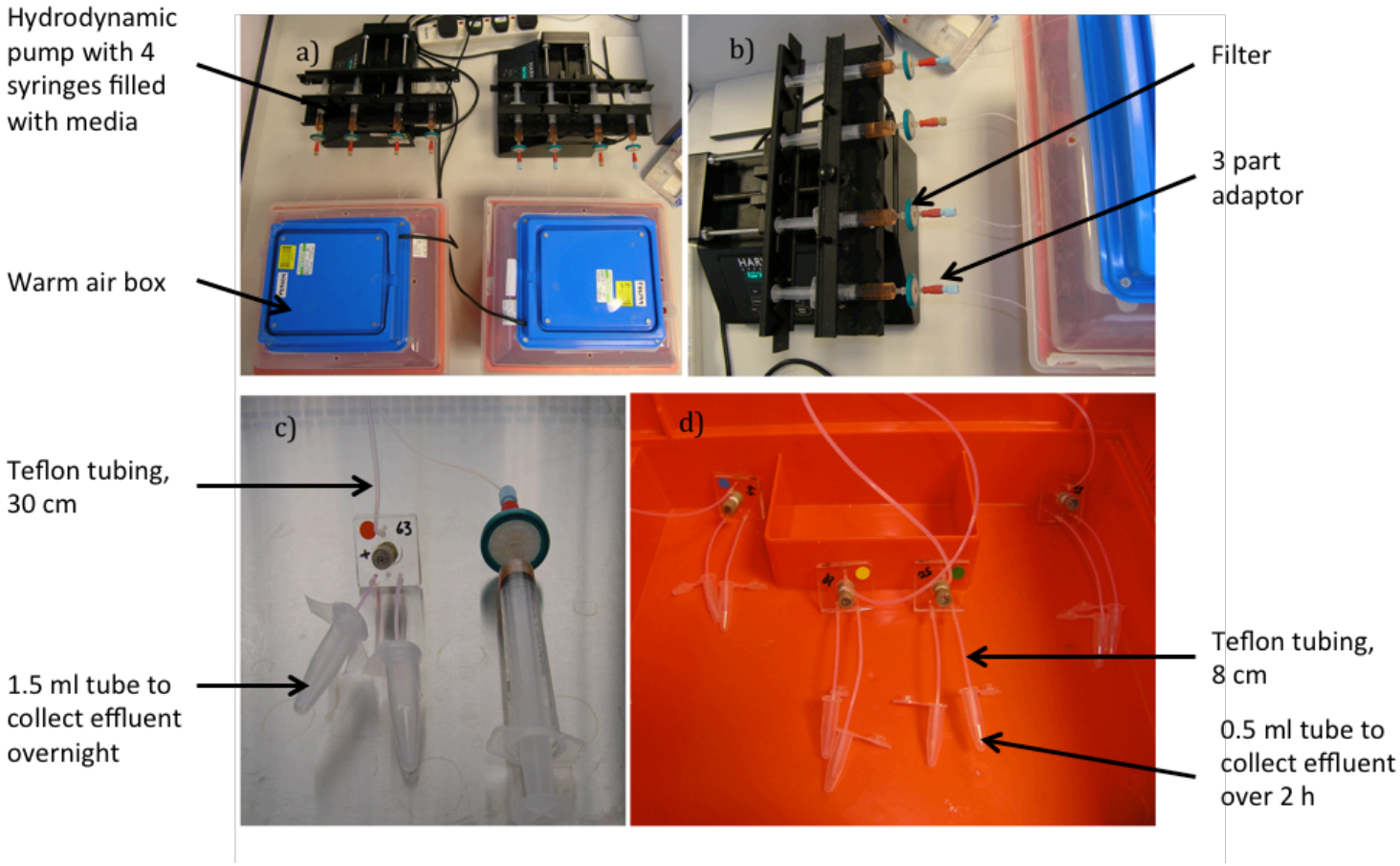



Figure 2.5 Components of the micro fluidic system, a),b) hydrodynamic pump, syringe, filter, 3 part adaptor, tubing and incubators, c) syringe, filter and tubing attached to device with outlet tubing and collecting tubes, d) 4 micro fluidic devices within the incubator.

### 2.3.3 Effluent Collection

Once the tumour was placed within the central well, the flow rate of media was reduced to 2 µl $min^{-1}$. The effluent collected over the first hour was collected and after this, effluent was collected in 2 hourly aliquots, each of 240 µl for up to 9 days.

## 2.4 Materials

### 2.4.1 Chemotherapy Agents

All drug preparation was carried out in aseptic conditions prior to addition to the standard supplemented culture media described in section 2.1.3. Chemotherapy agents were added to the micro fluidic system via the buffer inlet following a 22 h incubation period with culture media alone when used.

#### *2.4.1.1 Cisplatin*

Cisplatin (TEVA, Castleford, West Yorkshire, UK) 1mg/ml solution for intravenous injection was used. Cisplatin concentrations of 1 µg/ml - 25 µg/ml were studied.

#### *2.4.1.2 5-Fluorouracil*

5-Fluorouracil (5FU, Mayne Pharma Plc, Melbourne, Australia) 25 mg/ml solution for venous injection was used. A 1 in 100 dilution was prepared by adding 50 µl 5FU to 5 ml phosphate buffered saline (10 mM phosphate, 150 mM sodium chloride, pH 7.2 to 7.3, Invitrogen Ltd, Paisley UK). 5FU concentrations of 1 µg/ml - 25 µg/ml were studied.

#### *2.4.1.3 Docetaxel*

Docetaxel (Taxotere®, Sanofi-Aventis, Guildford, Surrey, UK) 80 mg in 2 ml solution for intravenous injection was used. The diluent 13% (v/v) ethanol in water for injection was added (fill range 6.96 – 7.70 ml) to give a final concentration of 10 mg/ml as per manufacturer's instructions. Docetaxel concentrations of 1.5 µg/ml - 37.5 µg/ml were studied.

### 2.4.2 Cell Death Analysis

To analyse cell death occurring within the tumour biopsy, the excretion of lactate dehydrogenase (LDH) into the effluent was quantified using a colorimetric cytotoxicity assay (Cytotoxicity Detection Kit Plus, Roche Diagnostics, Burgess Hill, West Sussex, UK). LDH is a stable cytoplasmic enzyme present in all somatic cells (Voet and Voet, 2004). *In vivo*, LDH catalyzes the inter-conversion of pyruvate and lactate with concomitant inter-conversion of nicotinamide adenine dinucleotide (NAD) and NADH +. It converts pyruvate, the final product of glycolysis to lactate when oxygen is absent or in short supply, and it performs the reverse reaction during the Cori cycle in the liver (Voet and Voet, 2004). It is rapidly released into the cell culture supernatant, i.e. effluent, when the plasma membrane is damaged and can therefore be used as a marker of cell death.

Using this assay, LDH activity is determined by a coupled enzymatic reaction whereby LDH firstly catalyzes the inter-conversion of pyruvate and lactate with concomitant inter-conversion of nicotinamide adenine dinucleotide ($NAD^+$) and NADH (Figure 2.6). Secondly, the tetrazolium salt Iodotetrazolium chloride (INT) is reduced to formazan by the transfer of 2 hydrogen ions and the actions of the catalyst (Fig. 2.6). The increase in the amount of enzyme activity in the effluent directly correlates to the amount of formazan formed during a limited time period. The formazan dye formed is water-soluble and shows a broad absorption maximum at approximately 500 nm.

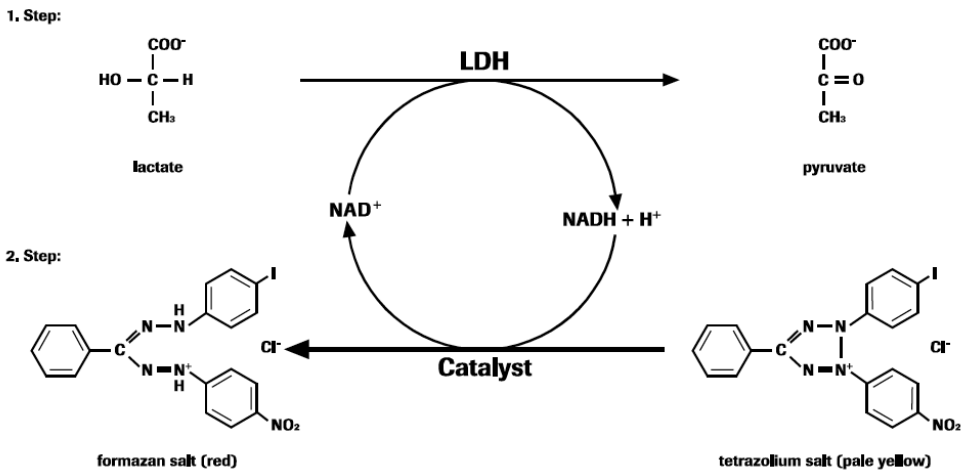


Figure 2.6 Diagram showing the assay of released LDH. In the first step, LDH reduces $NAD^+$ to $NADH^+/H^+$ by oxidizing lactate to pyruvate. In the second enzymatic reaction 2 hydrogens are transferred from $NADH^+/H^+$ to the yellow tetrazolium salt INT (2-[4-iodophenyl]-3-[4 nitrophenyl]-5-phenyltetrazolium chloride) by a catalyst.

The assay was carried out according to the manufacturer's protocol; 50 µl of 'reaction mixture' (lyophilizate, stabilized Diaphorase/$NAD^+$ mixture with INT and sodium lactate) was added to 50 µl tumour effluent per well in duplicate for each aliquot. Following incubation at 37 °C for 30 minutes and then the addition of 25 µl of 'stop' solution (1M Hydrochloric acid to stop the LDH reaction as per manufacturer's protocol), the absorbance of the samples was determined at 492 nm with a reference of 620 nm (to allow for plate differences and imperfections). The absorbance blank was set on the relevant media. The results are expressed as an average determined from duplicate samples and expressed per µg of tumour tissue used in each micro fluidic device. An example of the calculations is shown below:

| | Sample 1 | Sample 2 | Total | Average | Control | Minus Control | Biopsy (mg) | Per mg | Per ug |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3.219 | 3.223 | 3.221 | 3.221 | 0.9215 | 2.2995 | 3.6 | 0.63875 | 638.75 |
| 2 | 1.55 | 1.479 | 3.029 | 1.5145 | 0.9215 | 0.593 | 3.6 | 0.16472 | 164.72 |
| 3 | 1.698 | 1.612 | 3.31 | 1.655 | 0.9215 | 0.7335 | 3.6 | 0.20375 | 203.75 |
| 4 | 1.093 | 1.114 | 2.207 | 1.1035 | 0.9215 | 0.182 | 3.6 | 0.05055 | 50.55 |

Table 2.7 Example of calculations of LDH activity for individual aliquots

### 2.4.3 Cell Viability Analysis

To analyse cell viability, a colorimetric cell proliferation assay using a water soluble tetrazolium salt, 4-[3-[4 - Iodophenyl] - 2 - 4 (4-nitrophenyl)- 2H- 5-tetrazolio-1, 3-benzene disulfonate (WST-1) (Roche) was carried out. When added to the culture medium, the stable tetrazolium salt WST-1 is cleaved to a soluble formazan by a cellular mechanism that occurs primarily at the cell surface (Berridge et al., 2005). This bioreduction is largely dependent on the glycolytic production of NAD(P)H in viable cells (Figure 2.7). WST-1 is a chromogenic indicator for the cell's bio-reducibility in viability assays as the amount of formazan dye present in the

effluent directly correlates to the number of metabolically active cells present in the culture system (Roche, 2007).

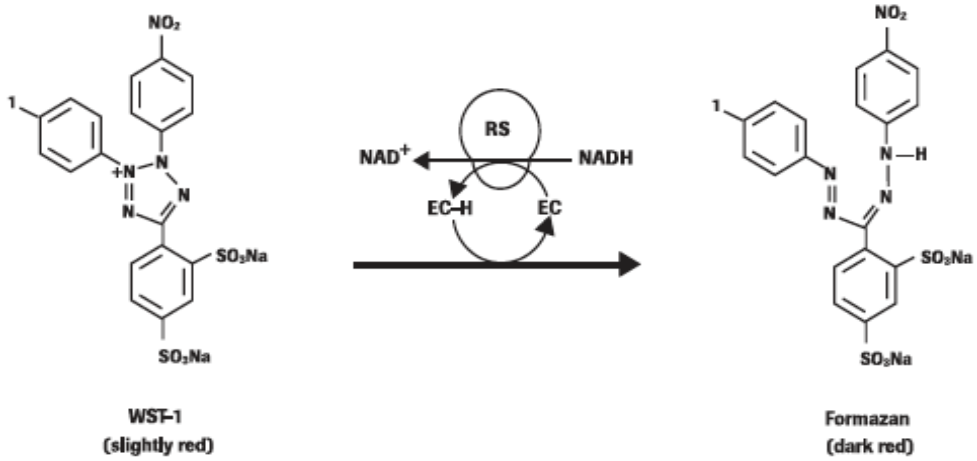



Figure 2.7 Diagram showing the conversion of the tetrazolium salt, WST-1, to formazan which occurs in viable cells. EC; electron coupling reagent, RS; mitochondrial succinate-tetrazolium-reductase system.

In viability studies, 10% v/v WST-1 was used and added to the culture media at 18 h of incubation. The assay consisted of a direct colourimetric analysis; 100 µl effluent was added to each well in duplicate for each aliquot and absorbance was measured at 450 nm with a reference of 620nm. The absorbance blank was set on the relevant media. The results are again expressed per µg of tumour tissue used in each micro fluidic device.

WST-1 has several advantages over other tetrazolium salts such as 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, (MTT) and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT). In contrast to MTT, which is cleaved to water-insoluble formazan crystal and therefore has to be solubilised after cleavage, WST-1 yields water-soluble cleavage products that can be measured without an additional solubilisation step (Berridge et al., 2005). In contrast to XTT, WST-1 is more stable and can therefore be used as a ready-to-use solution. It should be noted that LDH and WST-1 based redox reactions are tetrazolium salt specific.

### 2.4.4 Histo-architectural Analysis

To visualize cell architecture within the tissue, fresh frozen sectioning and haematoxylin and eosin (H&E) staining was carried out on tumour prior to, and following incubation within the micro fluidic device at 2 and 4 days. H&E staining is commonly used to differentiate tumour from normal tissue (Bancroft and Stevens, 1996). Haematoxylin, a basic dye, is principally employed to demonstrate nuclear structures, through a blue-black staining. Eosin, an acidic dye, has affinity for positively charged structures such as mitochondria, staining these areas pink or orange (Young et al., 2000).

The tumour biopsy (5-10 mg, approx 4mm$^3$) was first embedded and covered with Tissue-Tek® (Sakura, Netherlands) on a 2 cm x 2 cm cork tile before immediate immersion in a liquid nitrogen cooled 2-methyl butane solution. Samples were then stored at -20 °C before sectioning and staining. Tissue sections of 12 µm thickness were prepared using a Microm HM505E cryostat and dry mounted on microscope slides (Colorfrost Plus®, Thermo Shandon Ltd, Runcorn, Cheshire, UK) which could then be stored again at -20 °C until staining.

H&E staining was performed in accordance with published protocol (Bancroft and Stevens, 1996). Sections from the edge, centre and 'intermediate' area of the tumour were selected for staining and allowed to air dry at room temperature for 10 minutes. The sections were then fixed with 10% (v/v) formalin for 10 minutes, stained with Delafield's Haematoxylin for 3 minutes before rinsing with running cold tap water. After rinsing for 10 minutes the tissue was dehydrated with sequentially higher concentrations of 25%, 50%, 75%, 85% and 95% (v/v) ethanol over 8 minutes. The tissue was then stained with 100% eosin for 1 minute before being quickly dipped in 95% (v/v) and then absolute ethanol. The tissue was finally placed in Histo-clear (Fisher-Scientific, Loughborough, Leicestershire, UK) for five minutes to remove any excess stain and non-specific stain and then mounted using Histomount™ (National Diagnostics, Hull, East Yorkshire, UK) using a coverslip and allowed to dry for 24 hrs at room temperature. The tissue was imaged by light microscopy and photographed.

# 3.0 Results

## 3.1 Optimisation Studies

### 3.1.1 Aims

- To determine the stability of LDH in the effluent when stored prior to analysis
- To assess cell **death** within HNSCC biopsies over a 4 day period of incubation using analysis of LDH in the tumour effluent
- To assess cell **viability** within HNSCC biopsies over a 4 day period using metabolism of the cell proliferation agent WST-1
- To detect the presence of any bacterial contamination in the tumour effluent

### 3.1.2 Introduction

The various methods of tissue culture have been outlined in section 1.8 and in particular, the current limitations of whole tissue culture techniques have been discussed. The relatively short-lived tissue viability seen in organ cultures and tumour spheroids has been demonstrated using various markers of cell death for example lactate accumulation, DNA strand breaks associated with apoptosis and necrosis and changes in glucose and adenosine triphosphate (ATP) distribution (Hirschhaeuser et al., 2010). The majority of these indices require termination of incubation for analysis. However, one of the major advantages of the micro fluidic perfusion technique is the constant removal of metabolic waste and the ability to collect aliquots of this waste for analysis at time intervals with no detriment to the tissue biopsy.

As described in section 2.4.2, LDH is a stable cytoplasmic enzyme that is rapidly released when the plasma membrane is damaged. It would therefore be expected to be rapidly detected in the tumour effluent after deliberate tissue insult or to be gradually excreted over time, proportional to the number of dying cells. The production of LDH and its presence in tissue effluent using a micro fluidic culture method has been used previously to evaluate cell death within rat liver

biopsies (Hattersley et al., 2008, van Midwoud et al., 2010a) rat heart tissue (Cheah et al., 2010) and human colorectal tumour biopsies (Webster et al., 2010). The induction of LDH excretion by use of lysis buffer to deliberately rupture the plasma membrane of viable cells at the end of an incubation period can be used both to demonstrate the tissue viability as indicated by the magnitude of LDH response after addition of detergent to the system and secondly, when added throughout the incubation process, to provide comparative levels attributable to maximal tissue death.

Similarly, the cell proliferation agent WST-1 is a water-soluble salt that can be analysed in the tumour effluent rather than requiring destructive intra-cellular extraction. The use of lysis buffer, as in the LDH studies can be applied to cause loss of viability, demonstrated by the marked reduction in WST-1 metabolism on its addition. Correlating LDH and WST-1 data was expected to show an inverse relationship

As the aliquots of tumour effluent were stored at +2 to +8°C for up to 9 days prior to assay, the stability of LDH over time needs to be assessed first. The LDH assay product literature advised that 'the cell-free culture supernatant can be stored at +2to +8°C for a few days without loss of LDH activity' however it was necessary to ensure that the LDH activity when assayed was representative of the true tumour effluent at the time of collection. Once the stability of LDH over time had been determined, the viability of tumour biopsies over a 4-day period was ascertained using LDH and WST-1 assays of the tumour effluent.

Although great care was taken to avoid any potential bacterial contamination within the system, there was always a risk of infection, either due to the tumour itself or during incubation. This could potentially confound the results as some or part of the production of LDH or metabolism of WST-1 could be attributed to bacteria. To demonstrate the absence of bacteria within the micro fluidic system, final aliquots of culture media were plated on nutrient agar and examined after incubation.

### 3.1.3 Materials and methods

Tissue samples were taken from those patients undergoing curative resection of their primary tumour or metastatic lymph nodes for proven HNSCC at Castle Hill Hospital after informed consent had been gained as described in section 2.1.2. Samples were processed as described in section 2.1.3 and incubated within the micro fluidic device as described with the addition of 10% v/v WST-1 or 10% v/v lysis buffer to the culture media as appropriate. Lysis buffer (10% v/v) was added at the end of the incubation period (68 h) except in those studies when it was used continually from the start of the incubation period.

To determine the stability of LDH activity within the tumour effluent, fluid was collected from 1 to 10 h of incubation, assayed at the time of collection, stored at +2to +8°C for up to 8 days and repeatedly assayed every 48 h.

For the identification of the presence of any bacteria in the tumour effluent, samples of effluent were plated on nutrient agar and incubated for 48 h days at 37°C.

### 3.1.4 Results

A total of 9 different fresh tumours were used for optimisation studies: with 8 chips run, on average, per tumour sample, for up to 4 days duration. The numbers of chips per experiment for each variable i.e. LDH analysis, WST-1 analysis or addition of lysis buffer, and the sub-site of the tumour used is summarised in Table 3.1. Also represented is the number of 'successful' chips. This refers to the number of chips in which the buffer flowed without blockages over the 4-day incubation period. An unsuccessful chip is one that blocks during the experiments or had insufficient media in-flow for longer than a 10 minute period. Data from any 'unsuccessful' chip was excluded from the final analysis.

| *Experiment* | *Sub site of Biopsy* | *LDH Set-up* | *LDH Successful* | *WST-1 Set-up* | *WST-1 Successful* | *Lysis Set-up* | *Lysis Successful* |
|---|---|---|---|---|---|---|---|
| 1 | Larynx | 3 | 2 | 2 | 1 | 2 | 1 |
| 2 | Larynx | 3 | 2 | 2 | 1 | 2 | 1 |
| 3 | Larynx | 3 | 2 | 3 | 2 | 2 | 0 |
| 4 | Oropharynx | 3 | 0 | 3 | 0 | 2 | 0 |
| 5 | Hypopharynx | 3 | 0 | 3 | 0 | 3 | 0 |
| 6 | Node | 3 | 1 | 2 | 1 | 2 | 1 |
| 7 | Node | 3 | 1 | 3 | 1 | 2 | 1 |
| 8 | Node | 3 | 3 | 2 | 0 | 2 | 0 |
| 9 | Node | 3 | 0 | 2 | 1 | 2 | 1 |
| **Total** | | **27** | **11 (41%)** | **22** | **7 (32%)** | **19** | **5 (26%)** |

Table 3.1 Summary of number of experiments, tumour sub sites and successful chips run for optimisation studies

### 3.1.4.1 Stability of LDH

Tumour effluent (from 4 independent tumours) collected between 1 and 10 h incubation was assayed in duplicates for LDH activity over 8 days and the results demonstrated in figure 3.1. The percentage change in absorbance is also shown; the average change being 4.1% from day 0 compared with day 8. As maintenance of LDH activity was observed, samples could be stored prior to analysis at the end of each experiment.

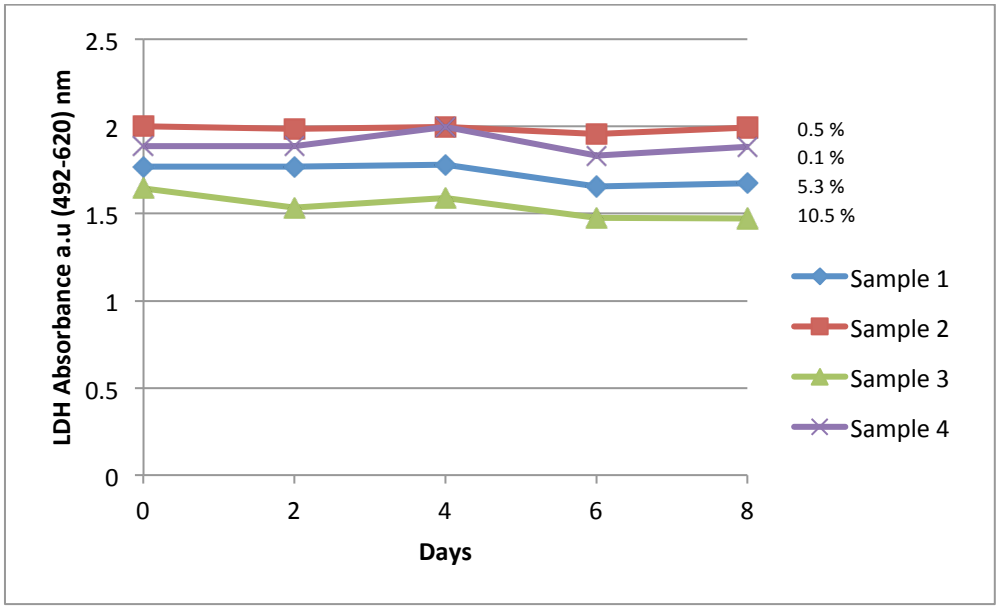



Figure 3.1 Graph to show the stability of LDH activity within tumour effluent over 8 days. Four different tumour samples are represented, each point representing the mean of a duplicate well, with the LDH calculated per piece of tissue (not standardised for weight) Percentage change from day 0 to 8 is shown for each sample.

### 3.1.4.2 Cell Death Analysis

The cumulative results of LDH release for 11 chips from 6 different tumours are shown in Figure 3.2. LDH is high in the tumour effluent after the 1st hour of incubation. This then falls over the next 2 h, remaining at a stable, minimal, level over subsequent hours until the addition of lysis buffer to the system at 68 h giving the anticipated increase in LDH release.

The results of adding 10% (v/v) lysis buffer throughout the whole incubation period are shown in Figure 3.3 for 5 chips from 5 different tumours. After a similar

initial LDH peak at 1-3 h incubation, levels of LDH fell but remained at a higher level than without the addition of lysis buffer throughout the period the incubation. Levels averaged 116.1 a.u. when lysis buffer was added, compared with 62.7 a.u. without between 20-26 h. Similarly, between 44-50 h, LDH levels averaged 93.6 when lysis buffer was present, compared with 57.3 without. There was also no observed 'peak' at the end when lysis buffer was present throughout the experiment.

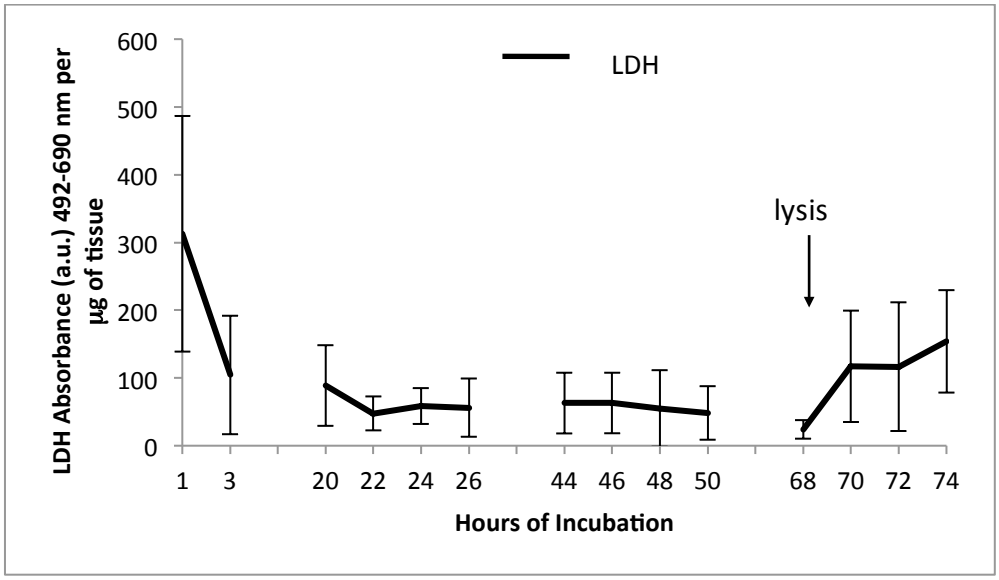



Figure 3.2 Graph depicting the pattern of LDH release per μg of tissue over 74 h incubation of fresh tumour biopsies (n=11 chips from n=6 tumours) with addition of 10%( v/v) lysis buffer at 68 h. Continuous line indicates LDH release as a mean of replicate experiments. Breaks in lines signify periods where samples were not collected. Error bars show standard deviation.

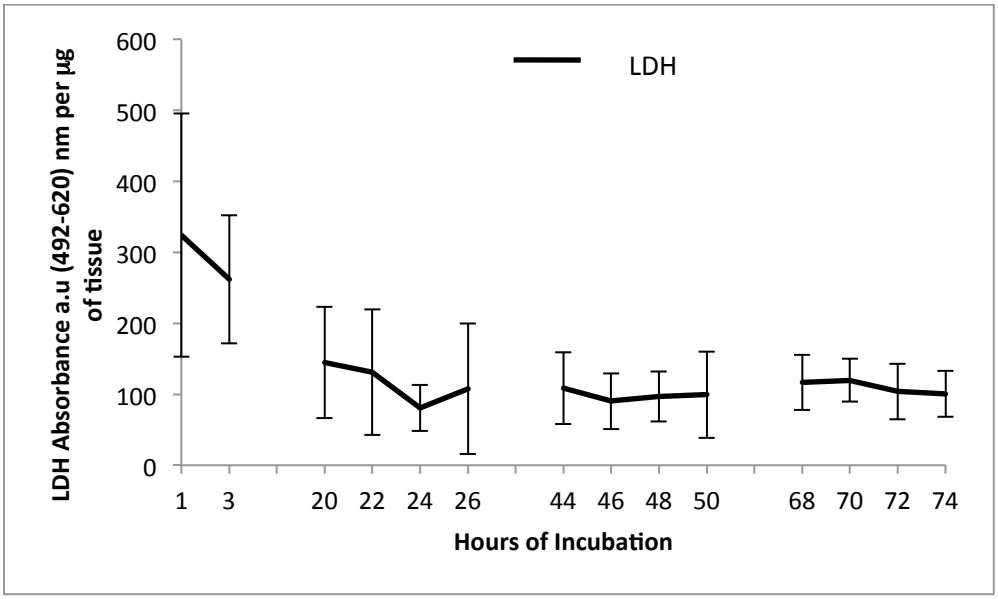



Figure 3.3 Graph depicting the pattern of LDH release per µg of tissue over 74 h incubation of fresh tumour biopsies (n=5 chips from n=5 tumours) with addition of 10% (v/v) lysis buffer throughout incubation. Continuous line indicates LDH release as a mean of replicate experiments. Breaks in lines signify periods where samples were not collected. Error bars show standard deviation.

#### *3.1.4.3 Cell Viability Analysis*

The cumulative results for WST-1 metabolism for 7 chips from 6 different tumours are shown in Figure 3.4. Two hours after the addition of WST-1, the presence of the metabolised salt in the tumour effluent can be seen to increase, with a maximum occurring in the second day following addition. The relatively high levels are maintained until the addition of 10% (v/v) lysis buffer at 68 h, which results in a reduction in its presence to minimal levels. The relatively large variation in results most probably reflects the inherent variability in metabolic activity of different tumours biopsies.

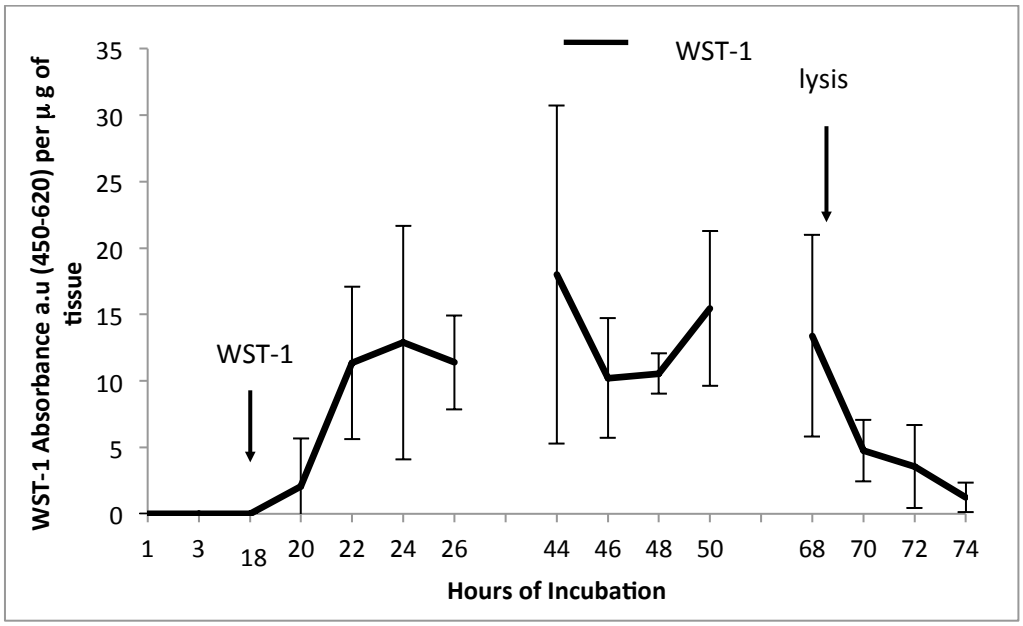



Figure 3.4 Graph depicting the pattern of WST-1 detection per µg of tissue over 74 h incubation of fresh tumour biopsies (n=7 chips from n=6 tumours) with addition of 10% (v/v) lysis buffer at 68 h incubation. Continuous line indicates WST-1 release as a mean of replicate experiments. Breaks in lines signify periods where samples were not collected. Error bars show standard deviation.

#### *3.1.4.4 Assessment of Bacterial Contamination*

Final aliquots of tumour effluent from 2 chips from experiments 5 and 7 plated onto nutrient agar and incubated at 37°C for 48 h did not show any bacterial presence. It is worth noting that all chips in experiment 3 did appear to become infected due to the fact that media became opaque and changed from pink to yellow colour in the effluent, reflecting a highly acidic environment due to extensive respiration and replication of the micro-organisms (Freshney, 2005). This opaqueness and media colour change was not seen in any of the other experiments.

### 3.1.5 Discussion

Initial studies on the stability of LDH when effluent was immediately stored at 4°C dictated that samples could be maintained at 4°C for up to 8 days prior to analysis. This meant that the LDH assay on all samples could be carried out the end of the incubation period, reducing the potential for variation between assays undertaken on different days.

Interpretation of the LDH studies, combined with evidence of WST-1 metabolism by the tumour demonstrates tissue viability within this *ex vivo* device. As predicted, the pattern of WST-1 metabolism, was inverseley related to LDH excretion, demonstrating continued viability and reduced cell death throughout the incubation period.

LDH has been used in several studies involving whole tissue culture within a micro fluidic device as a marker of cell death (Hattersley et al., 2008, van Midwoud et al., 2010a, van Midwoud et al., 2010b, Webster et al., 2010, Cheah et al., 2010). A similar trend in the pattern of LDH release seems to exist, in that a relatively high percentage of LDH leakage was found at the start of incubation. Van Midwoud et al. (2010a) estimated this amount of leakage to be equivalent to around 9% of the total LDH, attributed to slicing and slice handling procedures. Although this was based on rat liver slices of 100 μm (6 cell layers) within a PDMS rather than glass based micro fluidic device, this trend generally seems comparable with the finding of the current study. This trend was also seen in rat and human heart tissue in a PDMS micro fluidic device (Cheah et al., 2010), additionally, the removal of oxygen, cesation of electrical stimuation or the addition of Triton-X100 resulted in a sharp LDH increase within 10-15 minutes, again verifying the viability of tissue prior to induction of damage.

Tissue viability within micro fluidic devices can also be inferred by the continued 'functioning' of tissue. Hattersley et al. (2008) demonstrated that albumin and urea were continually synthesised and released into the effluent by rat liver tissue within a micro fluidic system. This also highlights an advantage of the micro fluidic-based approach; tissue function can be observed as a dynamic process, whereby changes in the tissue microenvironment can be studied by real-time evaluation of changes in the effluent. This is potentially an extremely powerful concept in relation to tumour biology.

Tumours are known to release a variety of factors as a means of manipulating their microenvironment to promote tumour growth and metastasis. The application of micro fluidic devices for the detection of tumour markers has already been appreciated, such as the work of Henry and colleagues (Henry et al., 2009), who developed a packaged micro fluidic cell for the multiplexed

electrochemical detection of cancer markers (described in section 1.9.2.2) however micro fluidic based tissue culture offers the possibility of real time evaluation of tumour response to changes in the microenvironment. To this effect, Webster et al. (2010) used the biomarker VEGF as a marker of tissue viability. VEGF, a fundamental neovascularisation factor (Tokunaga et al., 1998), is expressed by tumours, in which its release can be modified by environmental manipulation. The generation of low gaseous supply to create a hypoxic environment has been used to stimulate tumours to express VEGF (Kuwai et al., 2003) and based on this principle, Webster and colleagues demonstrated increased VEGF release of colorectal tumour biopsies in response to induced hypoxic conditions compared with normal tissues, with a reduction of VEGF release on return to normoxia.

Although the measurement of tumour related factors expressed in the effluent has not been evaluated in this study, future work could be undertaken on stored effluent from HNSCC, subject to ethical approval. Such work could be carried out on the effluent from viability studies and the same principle applied to those samples from tumour treated with chemotherapy agents as described in subsequent sections. Although in HNSCC no 'useful' secreted biomarkers have been identified as predictors of chemo/radiosensitivity (Dietz et al., 2010), surrogate markers such as VEGF could provide an interesting alternative until such factors are determined.

As shown in table 3.1, only around one third of chips were 'successful', i.e. ran throughout the entire incubation period. The most commonly encountered problem was blockage and therefore the cessation of flow of effluent. This was thought to be due to pieces of tumour detaching from the biopsy and blocking the downstream channel. This may be improved by modifying the chip; changing the channel dimensions could potentially reduce shear forces on the tumour biopsy (and therefore reduce the amount of detachment) and an increase in channel diameter could reduce the potential for blockage. Care would however have to be taken to ensure that any changes were not at the expense of tumour viability, as the micro channel flow characteristics and therefore diffusion properties would alter.

Another problem was leakage of buffer/effluent from around the tubing inlet/outlet ports. Although not as common a problem as device blockage, a slight adaptation in the methods of tubing attachment on to the device could reduce this

problem. Further work could focus on the development of a 'second generation' microchip to address these engineering/fabrication concerns.

## 3.2 Comparative Studies on Fresh and Frozen Tissue

### 3.2.1 Aims

- To compare cell death and viability in fresh and frozen primary tumour biopsies.

### 3.2.2 Introduction

The optimisation studies demonstrated that the micro fluidic system could maintain fresh pieces of HNSCC tumour for up to 4 days. The ability to store pieces of tumour prior to use (i.e. as snap frozen samples) is advantageous if biological properties are preserved and can increase the clinical applicability of this system. Successful cryopreservation would offer a possibility to store tissue biopsies, forming a tissue bank, from which tumours could be tested at any desired time-point. This is of particular use in HNSCC, in which the supply of newly presenting tumours is limited and irregular. The following experiments were therefore undertaken to determine tumour viability following cryopreservation.

The loss of cellular viability caused by rapid freezing of biological tissues using various cryoprotectants is well documented. Potential problems include intracellular ice formation and cellular toxicity attributed to cryotherapy agents (de Graaf et al., 2007), however such analysis is usually based on thawing of samples and analysis after a period of incubation. Studies were therefore undertaken to determine whether tissue viability, as measured by LDH excretion with immediate incubation via a micro fluidic-based approach on thawing, could be preserved in small biopsies.

### 3.2.3 Materials and Methods

Tumour samples were transported as described in section 2.1.3. After transportation of the tissue sample to the laboratory, half of the tissue was processed and immediately placed in the micro fluidic system for incubation (within 15 minutes of surgical dissection). The other half was snap frozen in cryoprotectant (within 15 minutes of surgical dissection) as described in section 2.1.3 and stored at -80°C for 2 weeks until thawing and subsequent incubation in the micro fluidic

device. The LDH activity in the tumour effluent was evaluated for the fresh and frozen tissue of each tumour.

### 3.2.4 Results

A total of 3 different tumours were incubated when fresh (1a, 2a, 3a) and following freezing and storage for 2 weeks (1b, 2b, 3b), with 4 chips run, on average, per tumour incubation, for up to 4 days. The numbers of chips per experiment (LDH analysis and WST-1 metabolism only) and the sub-site of the tumour used are summarised in Table 3.2. Also shown, is the number of 'successful' chips. This refers to the number of chips that ran successfully throughout the 4-day incubation period.

#### *3.2.4.1 Cell Death Analysis*

The cumulative results of LDH release for 10 chips (4 fresh, 6 frozen) from the 3 different tumours are shown in Figure 3.5. LDH is maximal in the tumour effluent after the 1st hour of incubation. The results demonstrate a similar LDH peak at the start of the experiment in both tissue groups. The two groups then show a very similar response with the amount of LDH released from the frozen tumour biopsy being slightly higher in the first 24 h and then falling to lower levels after this. Both groups responded to the addition of 10% (v/v) lysis buffer in a similar fashion; LDH release increased. This was greatest in the fresh group, presumed to represent the greater number of viable cell present at this time.

| *Experiment* | *Sub site* | *LDH Set-up* | *LDH Successful* | *WST-1 Set-up* | *WST-1 Successful* |
|---|---|---|---|---|---|
| 1a | Node - fresh | 2 | 1 | 2 | 1 |
| 1b | Node - frozen | 2 | 2 | 2 | 1 |
| 2a | Larynx – fresh | 2 | 1 | 2 | 1 |
| 2b | Larynx - frozen | 2 | 2 | 2 | 1 |
| 3a | Larynx – fresh | 2 | 2 | 2 | 2 |
| 3b | Larynx - frozen | 3 | 2 | 2 | 0 |
| **Total** | | **13** | **10 (77%)** | **12** | **6 (50%)** |

Table 3.2 Summary of number of experiments, tumour sub-sites and successful chips run for fresh *versus* frozen studies

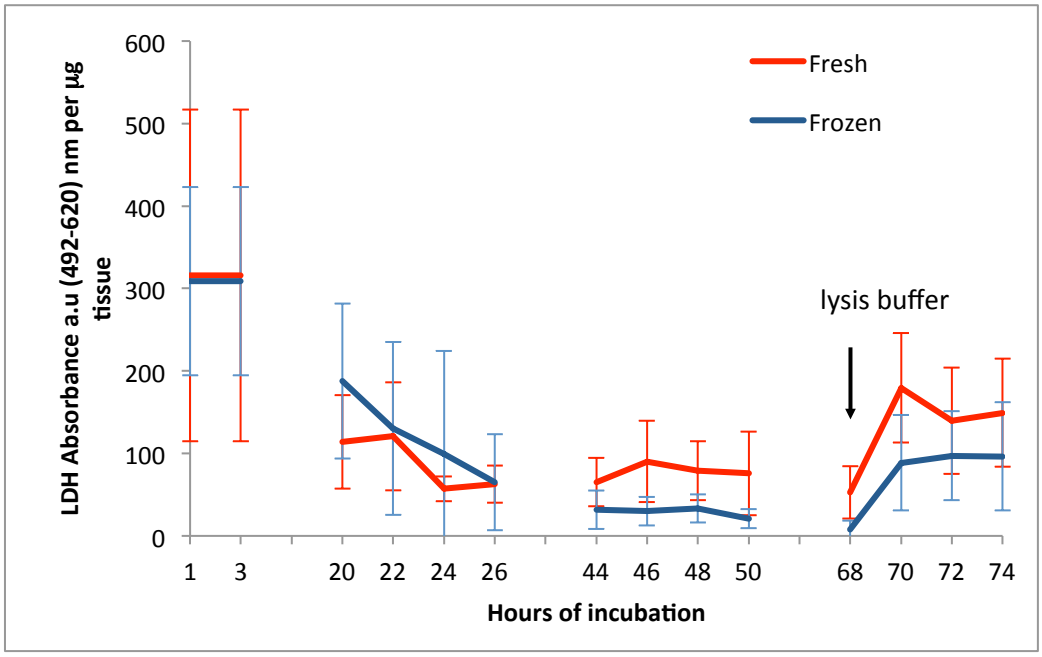



Figure 3.5 Graph depicting the pattern of LDH release per µg of tissue over 74 h incubation of fresh (n=4 chips from n=3 tumours) and frozen (n=6 chips from n=3 tumours) tumour biopsies with addition of 10% (v/v) lysis buffer at 68 h incubation. Red line indicates LDH release from fresh tumour, blue line from frozen. Breaks in lines signify periods where samples were not collected. Error bars show standard deviation.

### *3.2.4.2 Cell Viability Analysis*

The cumulative results for WST-1 metabolism for 6 chips (4 fresh, 2 frozen) from the 3 tumours are shown in figure 3.6. Two hours after the addition of WST-1 at 18h of incubation, the presence of the metabolised salt in the tumour effluent can be seen to increase in both of the tissue groups but more so in the fresh. At the later time periods levels continue to increase with time in both groups, with the fresh tissue reaching a greater maximal peak than the frozen until the addition of lysis buffer at 68 h that results in a reduction in WST-1 release returning to minimal levels in both groups.

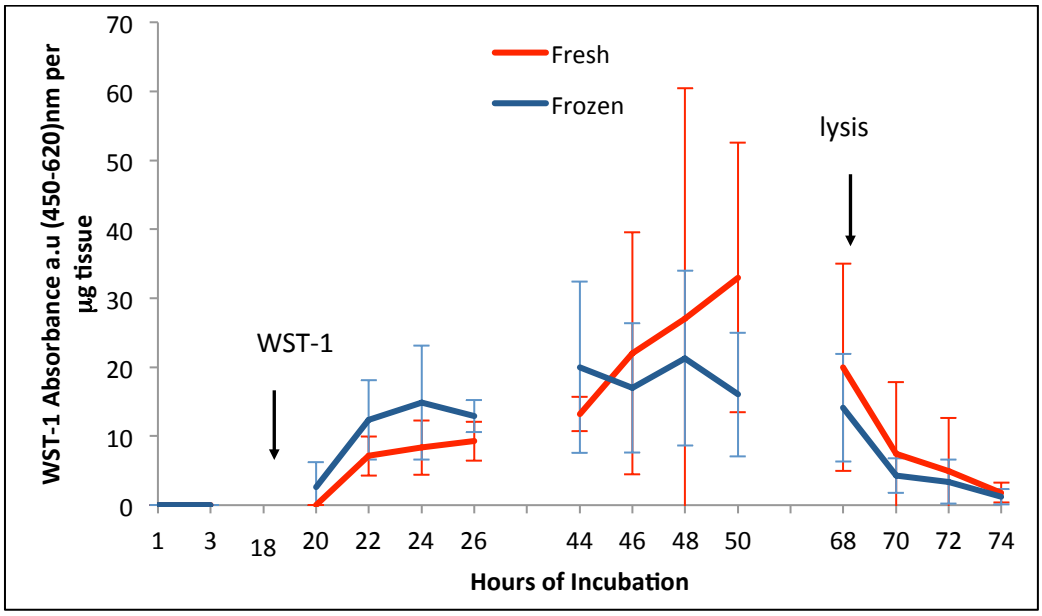



Figure 3.6 Graph depicting the pattern of WST-1 metabolism per µg of tissue after addition at 18 h incubation over 74 h incubation of fresh (n=4 chips from n=3 tumours) and frozen (n=2 chips from n=2 tumours) tumour biopsies with addition of 10% v/v lysis buffer at 68 h incubation. Red line indicates WST-1 release from fresh tumour, blue line from frozen. Breaks in lines signify periods where samples were not collected. Error bars show standard deviation.

### 3.2.5 Discussion

The release of LDH release from fresh tissue differed only slightly compared with the release from frozen tissue, with more LDH released from the frozen tissue in the first 24 h presumably due to increased damage associated with freezing and thawing. Additionally, less LDH was released from the frozen tissue on the addition of cell lysis buffer at the end of the experiment to rupture the membranes of any remaining viable cells. This suggests that frozen tissue had a reduced number of viable cells at the end of the experiment, presumably as there was increased cell degradation at the beginning of the incubation period associated with freezing and thawing. What proportion of cell death can be attributed to the cryopreservation process and how much cell viability, or 'rejuvenation' can be attributed to the micro fluidic process remains to be determined. The pattern of WST-1 metabolism would also suggest this; metabolism was greatest in the 'fresh' samples, although the overall trend similar, with both groups showing a reduction to a similar level after

the addition of lysis buffer. Of note, the sample numbers used in the WST-1 were lower than for LDH studies. This explains the larger standard deviation displayed in Figure 3.6. An increased number of repeats would enhance the validity of he LDH and more importantly the WST-1 studies above.

Many different techniques for cryopreservation exist and can be broadly categorised in to ultra-rapid freezing and thawing, controlled-rate freezing, freezing with non-penetrating polymers, vitrification, and equilibrium freezing (Meryman, 2007). In this study, ultra-rapid freezing was used for cryopreservation, as in the study of whole rat liver biopsies by Hattersley et al. (2008). This was however criticised by van Midwoud et al. (2010a) in their study of rat liver slices maintained in micro fluidic devices, stating that fresh tissue slices are necessary for proper viability and metabolic function. Undoubtedly some viability and metabolic functioning will have been lost during the cryopreservation and thawing process and perhaps this may have been optimised by a trial of various techniques and protocols, but importantly, the overall trend in LDH production and WST-1 metabolism was reproducibly maintained in the frozen tissue biopsies. As the trend in LDH release was maintained, it can therefore be argued that frozen tissue may be used within the micro fluidic device making a widely applicable platform technology as a way of measuring the *relative* changes in LDH release/WST-1 production attributed to different treatment regimens. This enhances the clinical applicability; tissue can be collected from the patient at the point of diagnostic biopsy and stored until diagnosis and clinical management have been decided upon, therefore allowing the micro fluidic device to be used as a testing platform for proposed treatment.

## 3.3 Histo-architectural studies

### 3.3.1 Aims

- To study the basic histo-architectural structure of HNSCC tissue following the incubation of fresh tissue within a micro fluidic device

### 3.3.2 Introduction

The qualitative analysis of tissue morphology adds to the evidence for preservation of tissue viability within the micro fluidic device. Morphological integrity of tissues is generally defined as preservation of general architecture including epithelial structures and their spatial relationships to stroma (Vaira et al., 2010). The most obvious histological feature of cell death (i.e. necrosis) is nuclear degeneration (Young et al., 2000). The first sign, known as pyknosis, results from condensation of the chromatin into a small, densely staining (Haematoxylin) mass. Later the nuclear material becomes fragmented (karyorrhexis) and ultimately breaks down with a loss of the nucleus altogether (karyolysis). At the same time, denaturation of cytoplasmic proteins results in the formation of an amorphous cytoplasmic mass, which stains intensely with eosin (bright pink). With the dissolution of the cell membrane, the cellular outline becomes increasingly difficult to distinguish (cytolysis) (Burkitt et al., 1993)

Taking cryostat sections of tissues enables the study of architecture at various levels through the biopsy. One of the disadvantages of traditional culture models is the limited size of tissue that can be cultured or maintained as a 3D construct. As previously discussed, unfavourable diffusion properties prevent the optimal diffusion of nutrients in, and metabolic waste out, of the centre of the tissue. It is hypothesised that the favourable diffusion properties associated with the laminar flow within micro fluidic devices, coupled with the use of relatively small tumour biopsies will avoid this problem.

### 3.3.3 Materials and Methods

Tumour biopsies were obtained and processed as described in section 2.1 and placed fresh into the micro fluidic device for perfusion. Tissue was either then removed at 48 h incubation or at 96 h incubation, at which point, snap freezing in

preparation for cryostat sectioning was performed as described in section 2.4.4. All pieces of tumour were stored at -20°C until sectioning, at which point cryostat sectioning, followed by Haematoxylin and Eosin (H&E) staining, was carried out. Sectioning of the tissue blocks resulted in between 15 and 35 slides with 2 to 3 sections of 12 μm thickness on each slide. The distance of section from the surface of the tumour was recorded for each section and categorised as 'edge' (outer third of the biopsy), 'centre' (core third) or 'intermediate' (area in between). Approximately 4 slides for each category per tissue block were selected for H&E staining.

### 3.3.4 Results

Three different tumours were used for these studies, in each case pieces of tissue were snap frozen before perfusion and following 48 h and 96 h of perfusion. The summary of sub sites, number of chips run and numbers of biopsy pieces snap frozen at each time point are shown in Table 3.3.

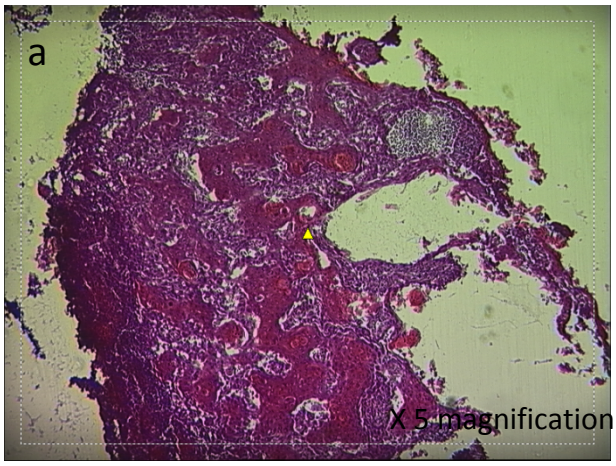
| *Experiment* | *Sub site* | *Before Perfusion* | *Chips Run* | *Tissue 48 h* | *Tissue 96 h* |
|---|---|---|---|---|---|
| 1 | Node | 2 | 4 | 2 | 1 |
| 2 | Node | 2 | 4 | 2 | 2 |
| 3 | Node | 3 | 5 | 2 | 2 |

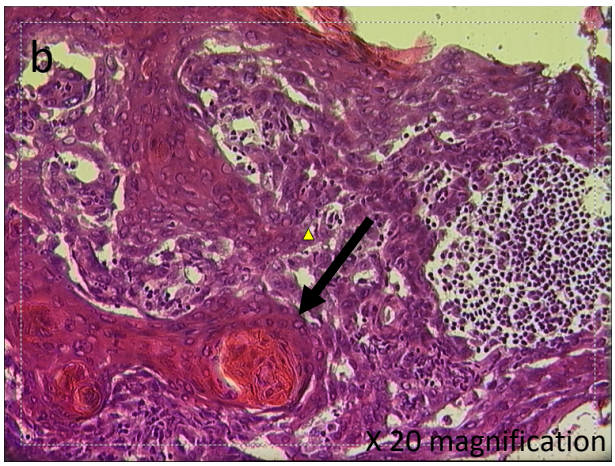
Table 3.3 Summary of number of experiments, tumour sub-sites number of pieces of tissue frozen at each time point for histo-architectural interpretation.

All tissue from experiment 2 was identified as connective/scar tissue on H&E staining, rather than HNSCC (confirmed with Dr Karsai, Consultant Histopathologist), therefore results are not shown or discussed.

Figures 3.7a, b, and c from 0 h, displaying areas of SCC and nodal tissue, show intact, viable cells with rounded nuclei and maintenance of cell-cell contact and cell membrane preservation. Figures showing tissue after 48 h (Figure 3.8) and 96 h (Figure 3.9) perfusion, do show some areas with loss of cell cohesion and nuclei degradation, however, large sections of apparently similar tissue structure of

epithelium and ECM to tissue at 0 h are present. There is no great difference in architecture between section from the edge of tumour (i.e. the biopsy surface and those from the core of the tumour biopsy (Figs 3.7, 3.8, 3.9), suggesting diffusion of nutrients into the centre is maintained. Additional figures for section from the tumour edge and intermediate areas (between the outer third and inner core third) are shown in Appendix 3.



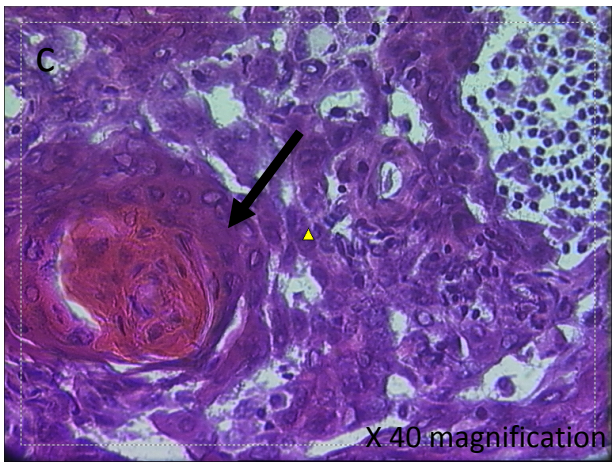
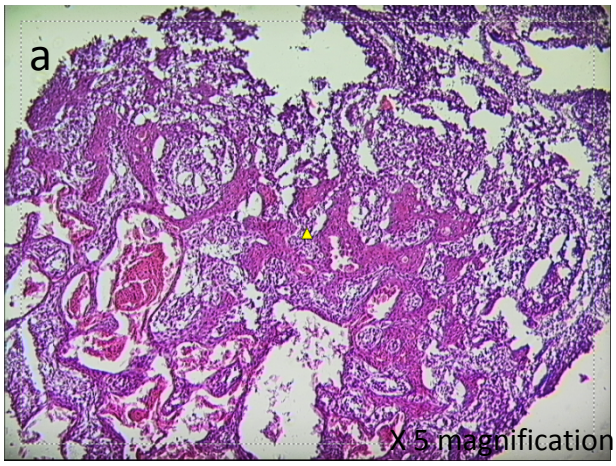
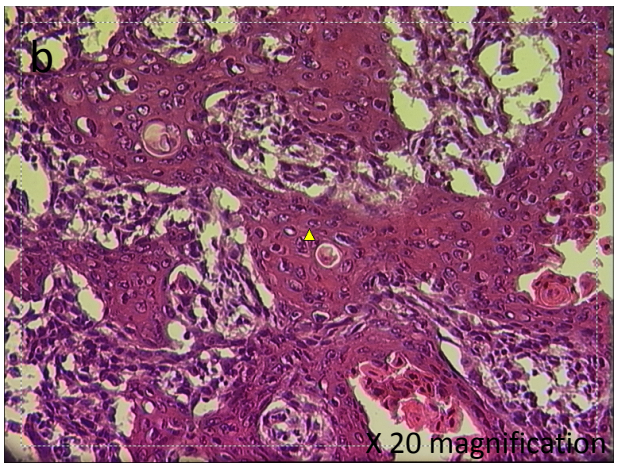




Figure 3.7 Image of central area of tumour/node prior to perfusion at a) x5, b) x20, c) x40 magnification. Areas of SCC (left side) with a characteristic keratin pearl (arrow) and a discrete area of nodal tissue far right. Representative of 5 tumour biopsies from 2 different tumours.



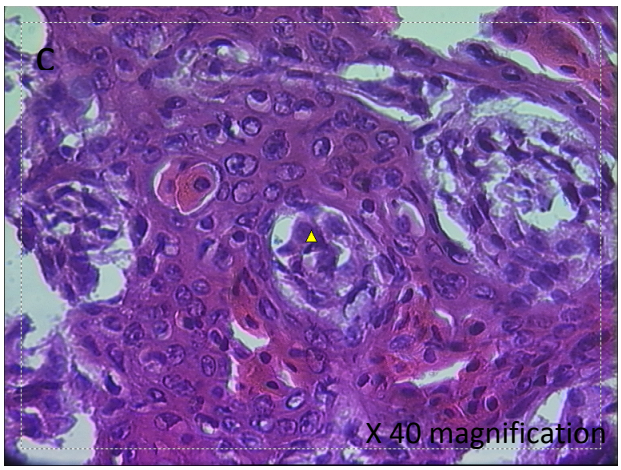
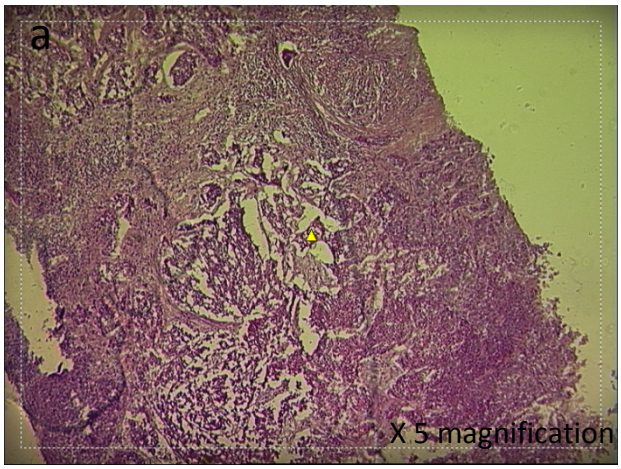
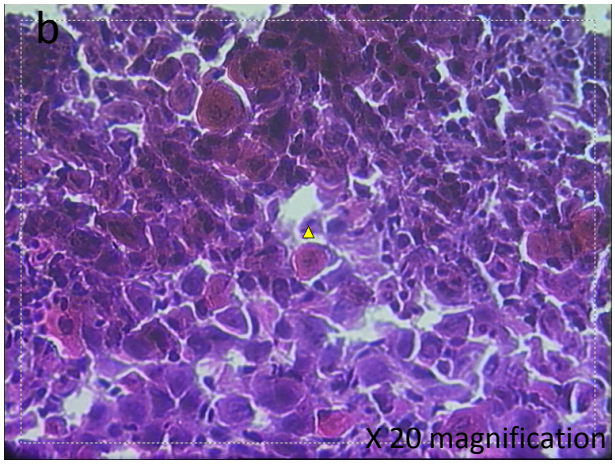




Figure 3.8 Image of central area of tumour/node at 48 h at, a) x5, b) x20, c) x40 magnification. Areas of cell viability with cohesion maintained. Areas of tissue separation evident but possibly due to the sectioning process. Representative of 4 tumour biopsies from 2 different tumours.



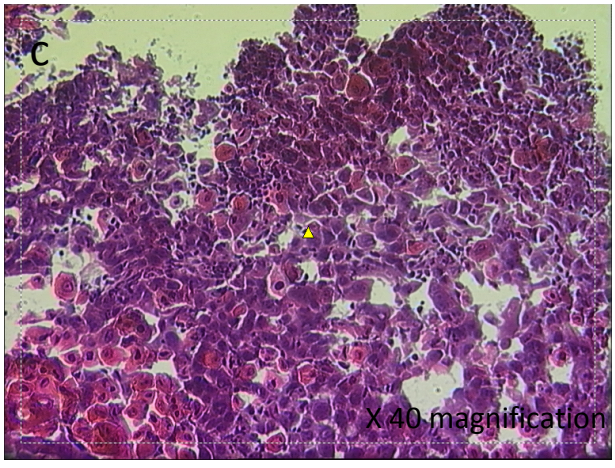




Figure 3.9 Image of central area of tumour/node at 96 h, at a) x5, b) x20, c) x40 magnification. Areas of intact nuclei identifiable with cohesion maintained in some places. Representative of 3 tumour biopsies from 2 different tumours.

### 3.3.5 Discussion

Histological analysis of tissue samples is one of the primary tools of diagnosis of HNSCC and has also been applied to the study of tissue within micro fluidic devices and alternative tissue perfusion systems to demonstrate the preservation of tissue architecture. Tissue architecture was preserved throughout the incubation and importantly this was evident in the centre or 'core' of the tissue biopsy. This is further evidence that the diffusion properties of micro fluidic devices, attributed principally to laminar flow, maintain sufficient nutrient delivery and waste removal throughout the tissue biopsy. Such findings were present in all samples subjected to histological examination (7 biopsies from 2 different tumours), demonstrating reproducibility and reliability in the micro fluidic techniques. It should however be stressed that these studies were only performed in n=2 tumours. Furthermore, comparison with non-perfused tumour (similarly left for up to 96) would add to the validity of the micro fluidic technique, but was not performed in this study.

This preservation of tissue morphology was also seen by Rambani et al. (2009), when culturing brain slices (>700μm) in micro fluidic perfusion chambers for 5 days. Perfused and unperfused tissues were cut into 20 or 50 μm thick transverse sections throughout the length and stained with H&E as in this study. They found that the overall structure, cell sizes and shapes of the perfused slices were comparable with those of the fresh tissue (baseline) slices that had been fixed immediately after cutting and stained identically, while the unperfused slices showed impaired cell health and poor overall structure. Notably, in these unperfused slices, the dendritic network was sparse and soma size comparatively smaller throughout the culture thickness, with these changes of poor health most significantly appreciated in the middle of the cultures. It was also found in the brain tissue that in the unperfused cultures, cell death occurred from the centre outwards.

The histo-architectural changes following perfusion have also been studied in rat liver tissue (Hattersley et al., 2008). Slices of 12 μm thickness from 4mm$^3$ pieces of tumour were stained with H&E and Lavacell™ fluorescence imaging used to evaluate tissue architecture after 70 h of perfusion within the micro fluidic device. In

sections of tissue taken throughout the tissue biopsies, the rounded appearances of the cell nuclei were maintained, as was the cell membrane and the hexagonal shape of the hepatocytes and the extracellular matrix. Fluorescent imaging of the liver tissue after staining with calcein demonstrated cell viability as shown by the retention of cleaved fluorescent probe calcein in the cytosol of viable cells. Conversely, the absence of staining with propidium iodide (which fluoresces red at 488 nm wavelength light when intercalated within DNA, having entered the cells through damaged membranes) demonstrated the absence of many non-viable cells. This appearance was reversed on the addition of 10% lysis buffer when staining indicated the presence of non-viable cells only.

To corroborate the observed results, an additional approach would be to use immunohistochemical (IHC) markers to demonstrate viability within the prepared tissue slices. This has yet to be applied to any micro fluidic-based tissue studies but has been applied to many alternative tissue culture methods. Vaira et al. (2010), acknowledging the benefits of whole tissue tumour culture to maintain the tumour microenvironment and its potential use as a predictor of chemotherapy response, used organotypic culture methods to maintain pieces of lung and colorectal tumour. Although these slices were only of 400 μm thickness, viability in a non-perfusate system for a 120 h incubation period was demonstrated by Ki-67 immunostaining for proliferative activity. The percentage of proliferating cells was approximately 82% in the first 24 h that reduced to around 62% on day 5. The authors maintain however that proliferative activity 'was found to be stable across all time points without a significant decrease up to 120 h'. Furthermore, examination of their stained images shows that with progression of time, proliferative activity becomes absent from the centre of the tissue slice and is only present at the tissue periphery. Apoptotic activity was also studied by TUNEL assay. This method detects DNA fragmentation from apoptotic signalling cascades by labelling the terminal end of nucleic acids by the addition of modified nucleotides that are secondarily labelled with a marker. Apoptotic activity was measured as 25% at 24 h, rising to 42% by day 5, however the distribution of apoptosis throughout the tissue slice was not shown.

The application of IHC staining to tissue cultured within a micro fluidic device is an interesting future area of research. As well as studying markers of proliferation,

IHC could be used to further investigate mechanisms of cell death and apoptosis within tumour tissue, for example Bcl/Bax pathways, as these factors have been extensively studies on conventionally prepared tissue (Gold and Kim, 2009).

## 3.4 Chemotherapy Studies

### 3.4.1 Aims

- To assess the effects of single and multiple chemotherapy agents on HNSCC biopsies within the micro fluidic system

### 3.4.2 Introduction

Optimisation and histo-architectural studies have demonstrated the maintenance of tissue viability and structure when perfused in the micro fluidic device. Furthermore, studies comparing the behaviour of fresh and frozen studies have demonstrated the preservation of biological properties when snap frozen in cryoprotectant and stored, increasing the clinical applicability of the approach. The ability to maintain a tumour with its intact *in vivo* structure within a microenvironment where flow characteristics are mimicked provides an ideal platform for the testing of a tumour's response to chemotherapy agents in a tissue-specific manner.

The evidence for the use of chemotherapy drugs in HNSCC is outlined in section 1.7, however individual tumours are known to be heterogenous in their response to commonly used agents and the problems of chemoresistance are too often seen in clinical practice. This highlights the need for a method of testing clinically relevant drugs on tissue biopsy in order to predict individual response.

When used clinically, the following local policy is adopted:

- Induction chemotherapy (given prior to surgery or radiotherapy) in locally advanced disease (docetaxel is excluded in nasopharyngeal carcinoma)
    - Docetaxel 75 mg/m$^2$ intravenous (i.v.) day 1, cisplatin 100 mg/m$^2$ i.v day 1, followed by 5FU 1 g/m$^2$ daily for 4 days i.v.
    - A total of 3 cycles given (at 3 weekly intervals)

or, if >65 or serious co-morbidities,

- Docetaxel 75 mg/m$^2$ i.v. day 1, cisplatin 100 mg/m$^2$ i.v. day 1, followed by 5FU 750 mg/m$^2$ daily for 5 days i.v.
- A total of 4 cycles given (at 3 weekly intervals)

- Concurrent chemotherapy (locally advanced HNSCC i.e. oropharyngeal/ hypopharyngeal, not commonly used for laryngeal malignancies)
    - Cisplatin 40 mg/$m^2$ i.v weekly, concurrent with radiotherapy for 6 cycles
    - Cetuximab 400 mg/$m^2$ i.v followed by Cetuximab 250 mg/$m^2$ concurrent with radiotherapy for 6-7 cycles if platinum-based chemotherapy treatment is contra-indicated.

To maximise the clinical applicability of the study, It was therefore decided to use cisplatin, 5FU and docetaxel alone and combinations of a) cisplatin and docetaxel, as administered on the same days in clinical practice and b) cisplatin, 5FU and docetaxel, as given overall. Cetuximab was not studied in the current investigation, as it is not a cytototoxic agent, but rather as a radiation sensitizer when given concurrently with radiotherapy (Bonner et al., 2006).

For chemotherapy drugs to be introduced into the micro fluidic system, some consideration is required regarding their dosage and distribution. Information regarding their pharmacokinetic properties is derived from animal models and early-phase clinical trials, allowing the mean drug concentration to be determined in plasma and in various tissues of the body, including tumours, as a function of time after administration. Having an appreciation of the plasma dose for example, and appreciating the distribution of chemotherapy agents within tumours *in vivo* will enable a comparable chemotherapy regimen to be used within the micro fluidic device.

In the *in vivo* setting, chemotherapy agents distribute within tumours, forming gradients from tumour blood vessels, that change with time, as the drug is cleared from the body (Minchinton and Tannock, 2006). The permeability of vessel walls influences drug penetration, but this is thought to be relatively insignificant in many tumours where blood-vessel fenestrations have been observed, i.e. the chaotic leaky vasculature allows ready transfer of chemotherapy drugs (Jain et al., 2002). Drugs penetrate normal tissues by both diffusion and convection, with a net flow of fluid from blood vessels balanced by resorption into lymphatics (Minchinton and Tannock, 2006). However, the lack of functional lymphatics commonly found in

tumours (Leu et al., 2000, Jain et al., 2002, Fukumura et al., 2010), especially more advanced malignancies, can lead to increased levels of interstitial fluid pressure, which in turn is likely to reduce convection and thereby inhibit the distribution of macromolecules (Jain, 1997).

The physicochemical properties of drugs (e.g. molecular weight, shape, charge and aqueous solubility) also influence the rate of diffusion through tissue. *In vivo,* drug penetration is also affected by consumption in proximal tumour cells (Tredan et al., 2007) as well as binding to tissue elements (both specific and non-specific target receptors) (Thurber et al., 2008). The penetration of paclitaxel (a similar agent to docetaxel) for example, is determined by multiple factors. Penetration is independent of biological changes in tumour cells induced by paclitaxel (e.g. ratio of EC and IC concentrations and drug binding to EC and IC macromolecules) (Minchinton and Tannock, 2006). Additionally, penetration is affected by time- and drug concentration-dependent biological changes (e.g. induction of apoptosis, enhancement of tubulin/microtubule production, and induction of Pgp expression) in histoculture models (Jang et al., 2001) and multicellular spheroids (Nicholson et al., 1997).

Although tumours require angiogenesis for growth, this is a dysfunctional process compared with the organised vessel formation in normal tissue. Furthermore, due to the rapid proliferation of tumour cells, vessels are forced apart reducing vascular density and creating a population of cells that are relatively distant (>100 μm) from blood vessels (Thomlinson and Gray, 1955). Close to the vessel wall, the tumour tissue is well supplied with nutrients and rapidly proliferates. Further from the blood supply, the concentrations of nutrients decrease and cells become quiescent apoptotic and eventually necrotic (Sutherland, 1988). In addition, extracellular pH decreases with increasing distance from blood vessels, Helmlinger et al. (1997) for example, finding the interstitial pH profile exhibited a mean drop of 0.32 units 10 μm (pH = 7.24) to 100 μm (pH = 6.92) away from the vessel wall. Constraining a cell mass within a square box would force linear microenvironment gradients to form perpendicular to a nutrient source (Figure 3.10) (Walsh et al., 2009) and although these linear microenvironment gradients would not mimic the complex radial and longitudinal gradients present in tumours (Dewhirst et al., 1999),

they would predictably reproduce the diversity of cell-types and environments surrounding blood vessels in tumours.

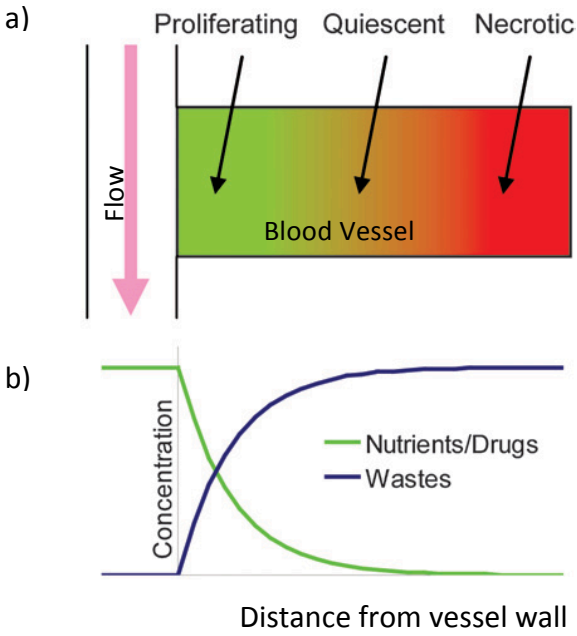



Figure 3.10 Diagram of tumour microenvironent gradients, A) the linear, observable microenvironment gradients in the micro fluidic device have a similar pattern to those surrounding blood vessels in tumours: proliferating (green), quiescent (transition), and necrotic (red). B) Conceptual concentration profiles of nutrients (green), drugs (green), and wastes (blue) around blood vessels can be emulated by micro fluidic devices; regions far from blood or culture medium are low in nutrients and drugs, and high in wastes. Adapted from Walsh et al., 2009.

Although the micro fluidic system does go some way to replicating the *in vivo* conditions, with so many variables to consider with regards to drug diffusion and penetration, dose response experiments were performed initially based on *in vivo* plasma dose levels.

With regards to cisplatin, peak plasma concentrations ranging from 2.5–5.3 and 0.22–0.73 μg/ml respectively (Vermorken et al., 1984, Vermorken et al., 1986), a mean observed peak concentration (Cmax) of 2.9 μg /ml and area under the curve (AUC) of 347 μg h $l^{-1}$ (Johnsson et al., 1996) have all been reported following doses of 100-mg/$m^2$ in HNSCC patients. In order to determine the optimal dose for use in

micro fluidic-based studies, based on these clinical studies it was therefore decided to use concentrations of 1, 5, 10 and 25 μg/ml.

A consistent target range of AUC for 5FU infusion-based regimens has been established as 20 – 25 mg/l $h^{-1}$ in studies largely of colorectal cancer patients, independent of administration modes (bolus vs infusion) and schedules (several hours to several days) (Ychou et al., 1999, Ychou et al., 2003, Di Paolo et al., 2008, Gamelin et al., 2008). This equates to a plasma concentration of 450-550 μg/l (Saif et al., 2009), although evidence suggests that when combined with other cytotoxic agents (e.g. cisplatin as in the case of HNSCC), a regime equating to an AUC of 25-30 mg/l $h^{-1}$ should be used (Ploylearmsaeng et al., 2006). This is achieved by generating a steady state plasma concentration of 0.55-0.65 μg/ml (Saif et al., 2009). Additionally, a dosage of 1000mg/$m^2$ generated an average Cmax of 2 μg/ml (Saif et al., 2009). Based on these findings, once again concentrations of 1, 5, 10 and 25 μg/ml were used in this study.

Studies of the administration of 1 h infusions of 100 mg/$m^2$ of docetaxel have demonstrated a Cmax of 4.7 μmol/l and an AUC of 5.69 μmol/l (Eisenhauer and Vermorken, 1998). Phase 1 drug studies using a similar dose reported a Cmax of 3.7 μg/ml and an AUC of 4.6 μg h $l^{-1}$ (Bridgewater, 2010). Concentrations of 1.5, 7.5, 15 and 37.5 μg/ml were therefore tested in the micro fluidic device.

### 3.4.3 Materials and Methods

Tumour biopsies were treated as previously described (section 2.1) The tumour was prepared into 4$mm^3$ sized biopsy pieces, snap frozen and stored prior to use as out-lined in section 2.1.3. On use, the tumour was prepared and micro fluidic devices were set and tested as in the viability studies (section 3.1). The appropriate chemotherapy regimen was added to the media, following 22 h incubation of media alone to allow the tumour to stabilise and the initial peak in LDH release to subside. Drugs alone and in combination were infused continually throughout the remainder of the incubation period.

Studies concerned with dosage of chemotherapy agent were performed over 4 days with effluent collection as in optimisation studies (section 3.1). Initial studies on different chemotherapy agents were performed over 5 days (short-term) as

viability studies had demonstrated the persistence of cell viability throughout this period. Finally studies were performed over a 9-day period (long-term) with effluent collection on days 1, 2, 5, 6, 7, 8 and 9 only, so that the continuing effects of the chemotherapy agents on days 5 to 9 could be elucidated. For these long-term studies, a fresh batch of chemotherapy agents was prepared and added to the micro fluidic system on day 5 of incubation to minimise reduction in drug efficacy associated with exposure to light and potential breakdown over a prolonged period. Results for the short and long term effects of chemotherapy were analysed as an average over a 24 h period. The effect of chemotherapy on cell death was determined by relative increase in cell death compared with the control and /or increasing drug concentration.

### 3.4.4 Results

#### *3.4.4.1 Dose response*

A metastatic lymph node from a tonsillar SCC was used to test the dose responses for all agents. Two micro fluidic chips were used as a control for each chemotherapy drug (a total of 6 chips). Four different doses were tested for each of the 3 agents in duplicate (24 chips). The results shown (Figure 3.11) are taken from one chip for each series as in most cases only one chip was successful.

All cisplatin concentrations caused an increase in LDH compared with control (Figure 3.11). This effect reduced over time, being maximal on days 2 and 3 of incubation. The greatest effect was seen on the addition of 5 μg/ml and above, therefore this concentration was used for further studies.

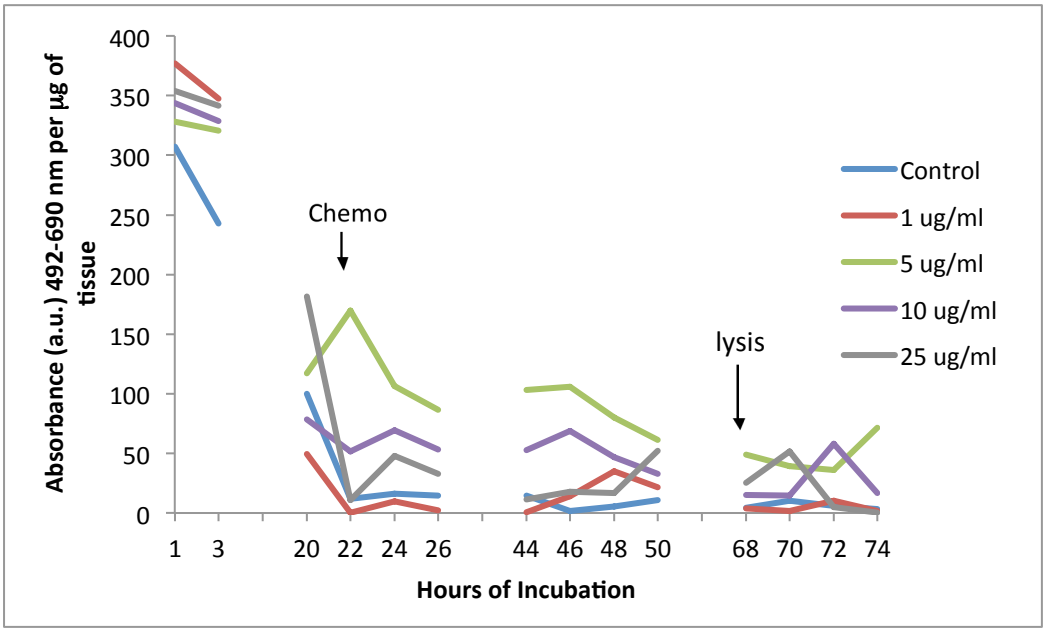



Figure 3.11 Graph depicting LDH release per µg of tissue after addition (22 h) and continued perfusion (to 74 h) with differing concentrations of cisplatin. 10% (v/v) lysis buffer was added at 68 h incubation. Each result is representative of one micro device only. Breaks in lines signify periods where samples were not collected.

On the addition of 5FU of any concentration, an increase in cell death, as measured by LDH release, was seen in a similar manner to cisplatin (Figure 3.12). Cell death was maximal on days 1 and 2 following addition and, overall, the resultant cell death decreased over time. Although at 25 μg/ml, cell death appeared to be maximal, the greater variability when compared with the other doses and missing points limited the data set. Again, 5 μg/ml was chosen as this value gave a cytotoxic effect and was within the plasma dose range found in the literature, accepting that higher doses may cause greater toxicity.

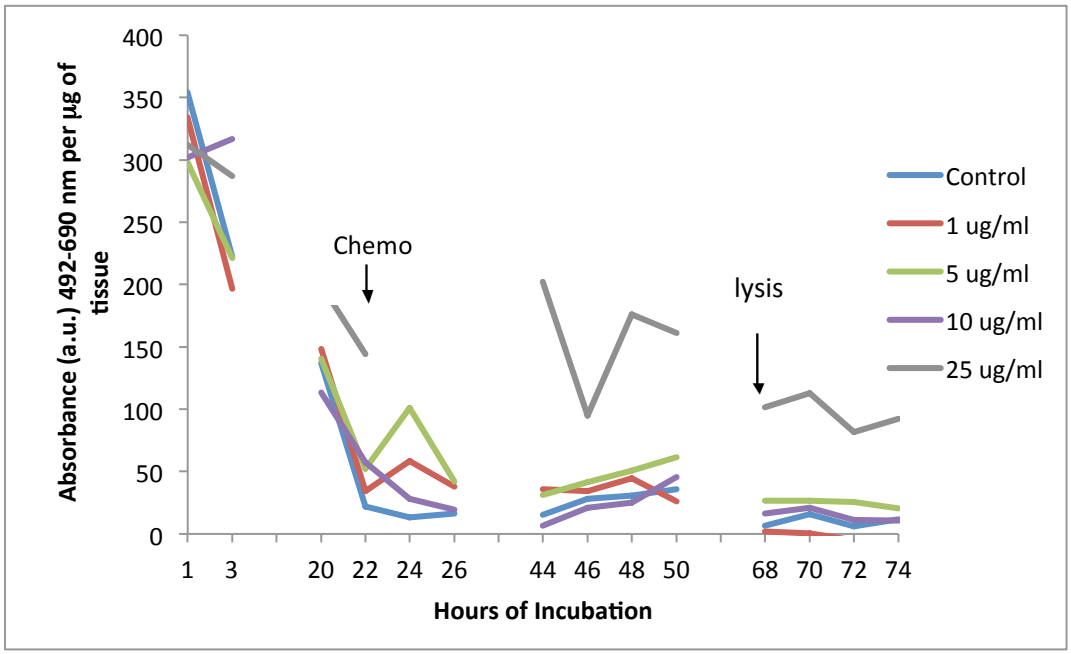



Figure 3.12 Graph depicting LDH release per μg of tissue after addition (22 h) and continued perfusion (to 74 h) with differing concentrations of 5FU. 10% (v/v) lysis buffer was added at 68 h incubation. Each result is representative of one micro device only. Breaks in lines signify periods where samples were not collected.

On the addition of docetaxel of any concentration, an increase in cell death, as measured by LDH release was seen (Figure 3.13). Cell death was maximal on days 2 and 3 following addition of drug, similar to the other chemotherapy agents. As with the other agents, the resultant cell death decreased over time. Although at 1.5 μg/ml, there was an apparent increase in cell death, this could have been related to interrupted flow (as was observed during this experiment) rather than an increased LDH due to the effects of the drug. It was therefore thought that the 7.5 μg/ml was a better representation of increased cell death, again this value being comparable with dosage found within the literature. This concentration was therefore used for further studies.

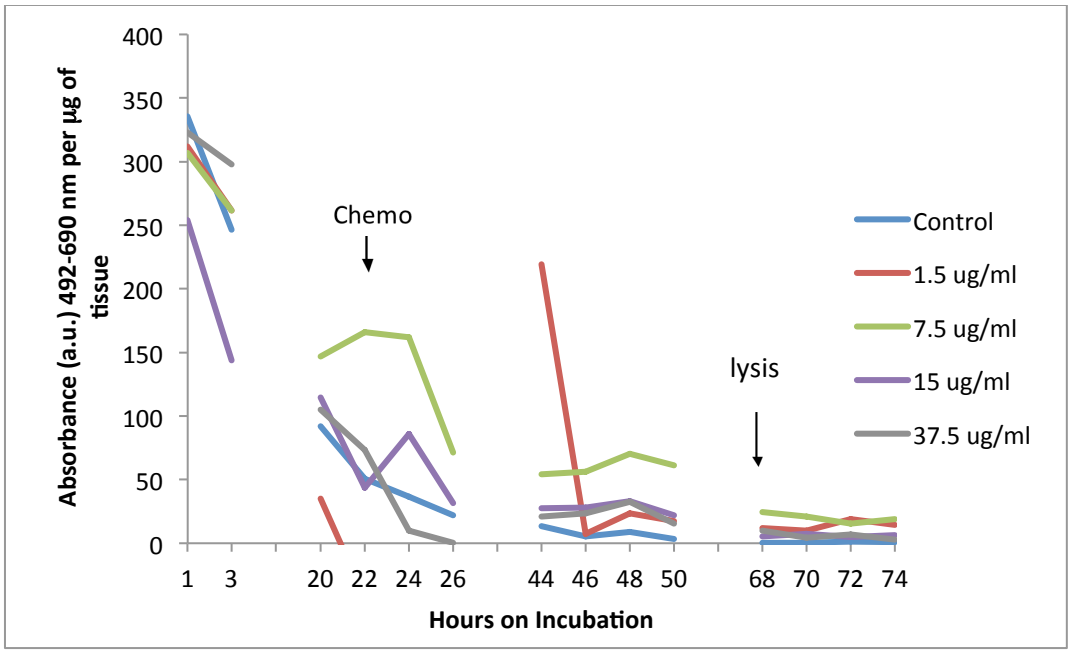



Figure 3.13 Graph depicting LDH release per μg of tissue after addition (22 h) and continued perfusion (to 74 h) with differing concentrations of docetaxel. 10% (v/v) lysis buffer was added at 68 h incubation. Each result is representative of one micro device only. Breaks in lines signify periods where samples were not collected and analysed.

#### *3.4.4.2 Short Term Chemotherapy*

Two different HNSCC metastatic nodes were assessed in duplicate for LDH response to chemotherapy agents when given alone and in combination when added after 22 h of incubation (day 1), perfused continually with the agent(s), and assessed over the following 4 days (days 2 to 5). The number of chips tested, successful and otherwise are shown in Table 3.4.

The increased cell death was most rapid following the addition of cisplatin, whereas the effect of docetaxel seemed to increase proportionally over time. The cell death associated with 5FU was more variable with a fairly rapid response and subsequent fall but evidence of an increase in cell death again on day 5. A similar pattern was seen on the addition of combined cisplatin and docetaxel; the early peak in cell death on day 2 perhaps being explained by the possible more rapid effects of cisplatin, with the late peak on day 5 by the slower effects of docetaxel causing an increase of cell death again with prolonged incubation.

The greatest increase in cell death is seen on the addition of all three agents. This was maximal on days 4 and 5 of incubation although a modest effect was seen on earlier days. As cell death seemed to increase with prolonged incubation for some of the agents and in particular, for combined agents, further studies were undertaken on the effects of cell death over a prolonged period of incubation.

The 2 tumours in this experiment seem to have behaved differently from those in section 3.4.4.1. In the dose response experiments cell death was greatest on the first 2 days of incubation and had reduced on the third day for all three agents to minimal levels despite the addition of lysis buffer at 68 h. In these short-term chemotherapy experiments, cell death was greatest on day 3 for cisplatin, continued increasing on day 3 and beyond for docetaxel and the combinations of agents and was variable for 5FU. Only the control tumour demonstrated a reduction in cell death with time. This variation could be accounted for by tumour heterogeneity. Additionally, as the tumour in the dose experiments was incubated for shorter time periods and was 'lysed' at 68 h, the results are not directly comparable.

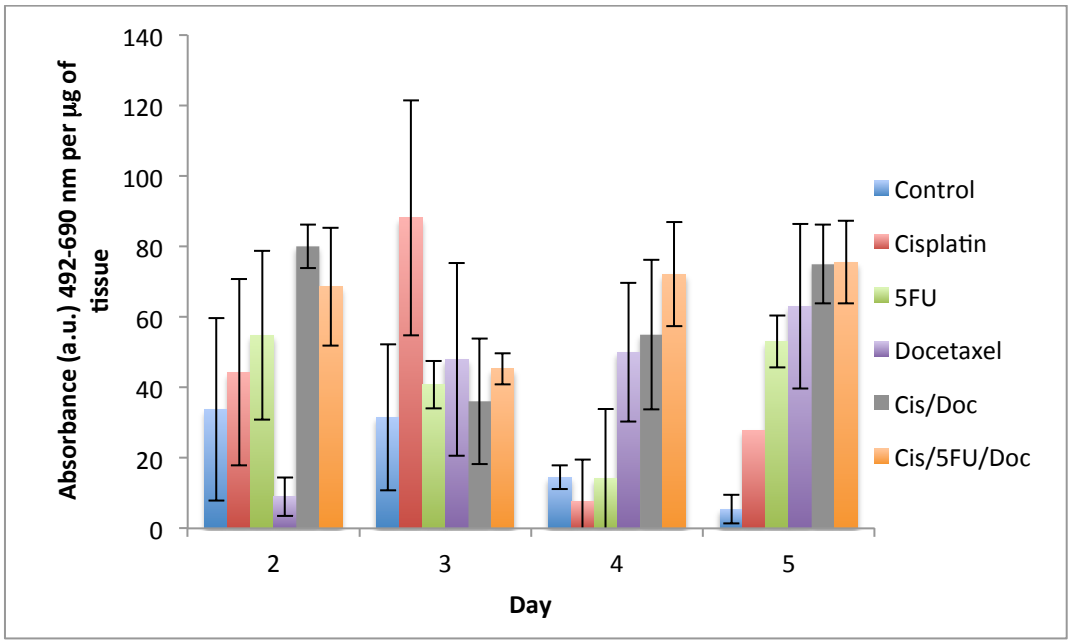



Figure 3.14 Graph depicting LDH release per µg of tissue of control (n=4 chips from n=2 tumours) and after addition (22 h) and continued perfusion (to 98 h) with cisplatin (n=3 chips from n=2 tumours), 5FU (n=3 chip from n=2 tumours), docetaxel (n=2 chips from n=2 tumours), cisplatin plus docetaxel (n=2 chips from n=2 tumours) and all 3 agents (n=5 chips from n=2 tumours). Error bars show standard deviation.

| *Experiment* | *Sub site* | *Control (Successful)* | *Cisplatin (Successful)* | *5FU (Successful)* | *Docetaxel (Successful)* | *Cisplatin + Docetaxel (Successful)* | *Cisplatin + Docetaxel + 5FU (Successful)* |
|---|---|---|---|---|---|---|---|
| 1 | Node 1 | 2 (2) | 1 (1) | 1 (1) | 1 (1) | 1 (1) | 2 (1) |
| 2 | Node 1 | 2 (0) | 1 (1) | 1 (1) | 1 (0) | 0 | 2(2) |
| 3 | Node 2 | 2 (2) | 1 (1) | 1 (1) | 1 (1) | 1 (1) | 2 (2) |
| 4 | Node 2 | 2 (0) | 1 (0) | 1 (0) | 1 (0) | 2 (0) | 2 (0) |
| **Total** | | **8 (4)** | **4 (3)** | **4 (3)** | **4 (2)** | **4 (2)** | **8 (5)** |

Table 3.4 Summary of number of experiments, tumour sub-sites and successful chips for short term chemotherapy studies. In experiment 4 a power cut resulted in the stopping of media in flow for a period of 2 hours on day 4. Results for this experiment were therefore not included.

#### *3.4.4.3 Longer Term Chemotherapy*

To assess the effects of the chemotherapy agents over a longer incubation (up to 195 h), two different metastatic lymph nodes were each subjected to chemotherapy agents in duplicate and are summarised in Table 3.5. Effluent was only collected on days 1,2 and 5 to 9 as information regarding the effects of chemotherapy agents on days 3 and 4 had been gained from the short-term experiments.

As found in optimisation studies and short term incubation with chemotherapy agents, in the control tumours, cell death decreases over time to minimal levels (Figure 3.15), and this remains the case at days 5 to 9. The addition of chemotherapy agents caused an increase in cell death on most days up to and including the final day of incubation. As in the short-term incubation studies, again cisplatin caused the most rapid increase in cell death, peaking on day 5 and then falling to moderate levels, then sustained though out the remaining incubation. Interestingly, the effects of docetaxel seemed to decrease proportionally over time after day 5, whereas in the short-term studies an increase had been seen between days 2 and 5, perhaps accounted for by tumour heterogeneity. In contrast, the effect of 5FU modestly increased from days 5 to 9. Again, an additive cytotoxic effect was seen on the addition of all three agents. This increased proportionally over time and was maximal on day 9.

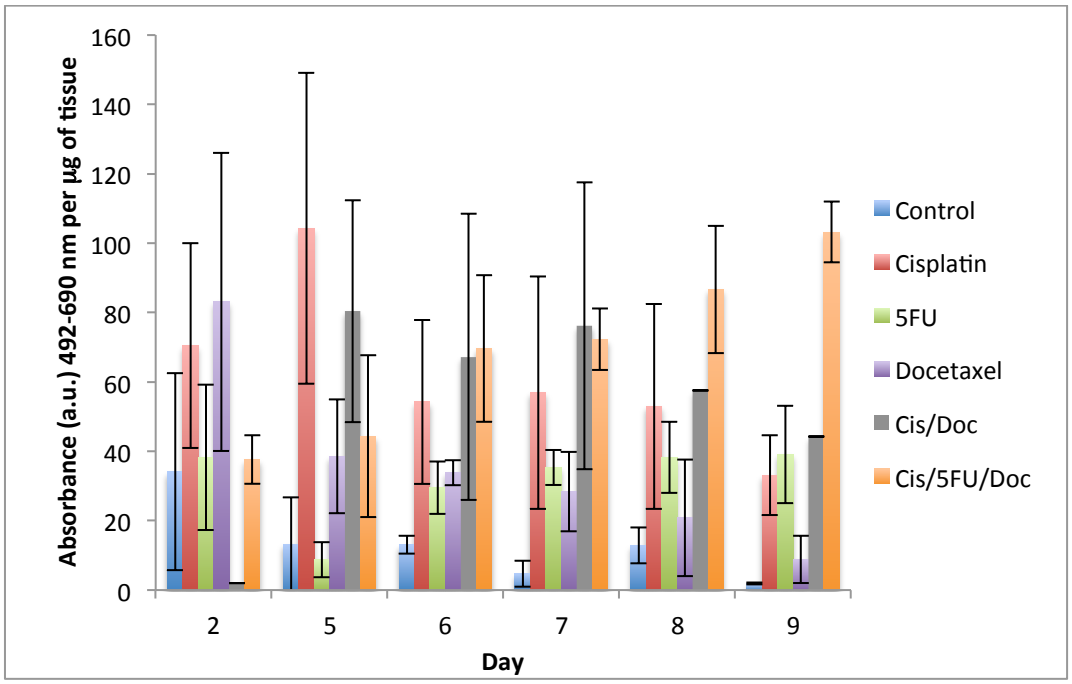



Figure 3.15 Graph depicting LDH release per µg of tissue of control (n=7 chips from n=2 tumours) and after addition (22 h) and continued perfusion (to 195 h) with cisplatin (n=7 chips from n=2 tumours), 5FU (n=5 chips from n=2 tumours), docetaxel (n=4 chips from n=2 tumours), cisplatin plus docetaxel (n=3 chips from n=2 tumours) and all 3 agents (n=5 chips from n=2 tumours). Error bars show standard deviation.

| *Experiment* | *Sub site* | *Control (Successful)* | *Cisplatin (Successful)* | *5FU (Successful)* | *Docetaxel (Successful)* | *Cisplatin + Docetaxel (Successful)* | *Cisplatin + Docetaxel + 5FU (Successful)* |
|---|---|---|---|---|---|---|---|
| 1 | Node 1 | 3 (3) | 2 (2) | 1 (1) | 1 (1) | 1 (0) | 2 (1) |
| 2 | Node 1 | 3 (2) | 1 (1) | 1 (1) | 1 (1) | 2 (2) | 2 (2) |
| 3 | Node 2 | 2 (2) | 2 (2) | 2 (1) | 2 (1) | 1 (0) | 2 (1) |
| 4 | Node 2 | 2 (0) | 2 (2) | 2 (2) | 2 (1) | 2 (1) | 2 (1) |
| **Total** | | **10 (7)** | **7(7)** | **6 (5)** | **6 (4)** | **6 (3)** | **8 (5)** |

Table 3.5 Summary of number of experiments, tumour sub-sites and successful chips for long term chemotherapy studies. A further experiment (experiment 5) with oropharyngeal SCC was attempted, however, due to stalling on the pumps on day 4 of incubation, the experiment was abandoned.

### 3.4.5 Discussion

These results show the utility of the micro fluidic device as a platform to test a variety of chemotherapy agents as single and combined therapies on individual tumour biopsies. Although the short and longer-term studies were only carried out on two different tumours in duplicate, results demonstrated a remarkable similarity in trend on the addition of the chemotherapy agents. This is the only study to date that has combined whole tumour culture within a micro fluidic device with the testing of chemotherapy agents.

This study is however limited by the number of experiments performed. Regarding the chemotherapy dosage experiments, only one chip was used for each drug dose and control. Ideally this should have been duplicated, especially as some of the chips exhibited variable flow patterns (and therefore questionable LDH levels). In addition, only one tumour biopsy was used for all experiments. As has already been alluded to, tumours demonstrate great heterogeneity in their response to chemotherapy agents. Although a seemingly appropriate response was seen with the addition of all chemotherapy agents in these experiments, how this would compare with different tumour sub sites or even tumours of the same sub site remains unknown. These experiments were used to determine the optimal chemotherapy dosage to be used in further experiments, and although the literature pertaining to *in vivo* plasma drug levels was considered, repeat experiments on different tumour sub sites should have ideally been performed and would have been if time had permitted.

Such limitations are also applicable to the short-term and longer-term chemotherapy studies. Two different samples of nodal tissue containing metastatic SCC were used for both experiments. Although the presence of nodal metastasis is often an indication for the addition of chemotherapy to a treatment regimen, the effect of chemotherapy agents on the primary tumour within a micro fluidic device remains unknown and is the ongoing study of other post-graduate students in the laboratory. Although these experiments were duplicated, further experiments are essential to demonstrate the robustness and reproducibility of this technique as well to investigate the tumour variability.

To investigate the cytotoxic effects of the various chemotherapy agents, LDH alone was used, whereas in the viability studies, the addition of WST-1 to the media fluid further validated the results. WST-1 was not used in the chemotherapy studies due to time constraints, however future studies would be of great interest. The additional inclusion of a measure of viability would strengthen the conclusions of the chemotherapy effects, as seen in the complementary behaviour of LDH and WST-1 in the initial validation study.

Another technique for assessing the response of tissue to chemotherapy agents would be to use IHC. Changes in protein expression of single cell cultures in response to chemotherapy have been studied in HNSCC as described in section 1.7 however these are yet to be demonstrated in tissue culture methods. Vaira et al. (2010) used changes in the PI3K/Akt signalling pathway as proof of concept of the chemotherapy effects in their *ex vivo* organotypic culture model. Untreated lung, prostate, and colon tumours showed constant levels of p-Akt andp-S6RP that decreased dramatically after *ex vivo* treatment with the PI3K inhibitor LY294002 in a dose-dependent manner. Such techniques enable the interpretation of these surrogate markers of effect.

Based on the methodology used for histo-architectural as described in section 3.3.3 tissue could be removed from the device post chemotherapy treatment and stained for potential prognostic markers. As described in section 1.7.1, the role of p53 and p16 in the cytotoxic action of cisplatin may have significance as prognostic markers to predict a tumour's chemosensitivity with status or indeed changes in expression of p53 and p16 following chemotherapy treatment *in vitro* of potential value for treatment planning (Yip et al., 2006). Other potential proteins of interest include the changes in expression of p53, Rb protein and p21WAF1/CIP1 protein with 5FU exposure (Lee et al., 2005, Liu et al., 2006) and the correlation of Bcl-2/Bax status with sensitivity to docetaxel (Kawakami et al., 1999). Examining these changes in protein expression (e.g. p16) with particular reference to HPV status would also be of interest, as well as differences in general chemosensitivity between HPV negative and positive tumours.

In addition, measuring changes in tumour-mediated factors in response to chemotherapy is an area of research with great potential to increase our understanding of drug/tumour interactions. As demonstrated by the work of Webster et al. (2010) on colorectal tumour biopsies within a micro fluidic device, the quantification of the tumour mediated factors VEGF in response to changes in the tumour microenvironment presents an alternative means of analysis. Although, no suitable, soluble biomarkers have been identified as of prognostic relevance in HNSCC as yet, the HNSCC expression of surrogate markers important in other solid tumours would be of interest.

# 4.0 Thesis Summary and Future Work

The initial aim of this thesis was to validate and optimise the micro fluidic system for the interrogation of HNSCC throughout a prolonged incubation period, by analysing markers of cell death and proliferation. This was achieved, with changes in LDH excretion into the tumour effluent and increases in WST-1 proliferation showing a reproducible trend, comparable with published literature on liver biopsies incubated in a similar manner (Hattersley et al., 2008, van Midwoud et al., 2010a). A total of 9 different tumours representing common HNSCC sub sites and metastatic nodal tissue were assessed for changes in LDH production and WST-1 proliferation within the device in various situations (buffer alone and with lysis throughout).

In this study, changes in tissue architecture were subjectively analysed; the changes associated with cell death and apoptosis were described in terms of the preservation of the cell nuclei and cell-to-cell cohesion. Although there was very little change in architecture (Dr Karsai, Consultant Histopathologist in Head and Neck Cancer, assessed 'no difference' in incubated samples compared to those immediately frozen), a more objective approach would be used in future studies given sufficient time and resources. For example staining techniques such as Lavacell™, based on the binding of the fluorescent calcein probe to the cytosol of viable cells as a marker of cell viability coupled with binding of the fluorescent propidium iodide to damaged intracellular DNA, as a demonstration of apoptosis, could be incorporated.

To confirm the advantages of the micro fluidic technique, changes in tissue architecture and viability need to be directly compared with tumour cultured by existing, commonly used, methods. As described in section 1.8 tissue culture techniques such as organotypic and histotypic culture are often employed. A major limiting factor regularly reported, particularly of whole tissue (organ) culture, is the lack of effective tissue perfusion. Micro fluidic-based tissue culture is most similar to organ culture techniques as both preserve the tumour microenvironment. Thus a systematic comparison of the tissue is required. As organ culture typically uses

smaller biopsies than the micro fluidic technique (e.g. 1mm$^3$ samples of UADT mucosa as used by Kleinsasser and colleagues (2004)), biopsies of various sizes of HNSCC tissue incubated using organ culture techniques (e.g. filter well inserts or grown on a stainless steel grid) should be compared to the 4mm$^3$ micro fluidic grown samples. Examining the histo-architectural changes of HNSCC tissue, taken from the same initial biopsy, cultured by these alternative methods will allow critical evaluation of the effectiveness of the micro fluidic system. In addition, it would be very interesting to perform an mRNA genome screen of tissue biopsies maintained in the micro fluidic device over time, as compared with tissue samples, and those maintained in static culture. This analysis would allow assessment of both "adaption" to culture in the micro fluidic device as well as key changes between culture methods. Obviously the mRNA screen would require verification at the protein level by Western blot or IHC.

The second aim of the work was to optimise the platform for studying chemotherapy administration in HNSCC. In these studies, the most suitable doses of chemotherapy agents to use on HNSCC within the micro fluidic system were determined. Although these experiments were, to some extent, limited by a lack of experimental replication, the fact that the tissue did respond in a seemingly dose dependent manner at concentrations comparable to those found *in vivo* is evidence of the ability of micro fluidic devices to mimic *in vivo* physiological conditions and tumour microenvironment. Furthermore, all of the tumours tested did respond to the chemotherapy agents, as predicted, by an increase in cell death compared with control. Such findings do not only demonstrate the role of tissue culture within micro fluidic devices but also show the potential integration of 'whole tissue' culture and pharmacological studies using micro fluidic based tissue culture.

As described in section 1.9.2.4 the application of micro fluidic technology to pharmacological studies has already been studied, with areas of interest including drug discovery and pre-clinical toxicology studies on multi-organ micro fluidic devices, pharmacodynamic studies and the analysis of chemotherapy resistance in cell lines. To date such studies are mostly based on cell cultures however the integration of whole tissue maintenance, with its enhanced ability to recreate the *in vivo* microenvironment of numerous tissue types, provides a highly applicable and

versatile platform for pharmacological studies. An area of particular interest is that of resistance to chemotherapy by HNSCC and potential molecular mechanisms involved. Using a micro fluidic-based approach, HNSCC tissue can be stratified by its cytotoxic response to chemotherapy and can be further evaluated by IHC for differences in expression of known key proteins e.g. p16 (Yip et al., 2006), p53, Rb and p21 (Lee et al., 2005, Liu et al., 2006) and Bcl-2/Bax (Kawakami et al., 1999).

The final aim of this thesis was to evaluate the potential of the micro fluidic approach for widespread clinical applicability. The response of individual tumour biopsies to single, and combination chemotherapy agents, although in its infancy demonstrated proof of concept. Undoubtedly these experiments need repeating, especially incorporating other HNSCC tumour sub sites, however the potential application includes the individualisation of chemotherapy treatment for not just HNSCC but all solid tumour types.

The potential for micro fluidic technology for automation and integration on to a portable device has already seen the development of devices for point-of-care diagnostic instrumentation (Herr et al., 2007). Ultimately, incorporating the findings of this thesis with the applicability of micro fluidic technology, an automated device could be developed to test individual patient biopsy samples in order to generate tumour specific response to chemotherapy. Once validated it is proposed that data generated from the micro fluidic interrogation of biopsies, obtained from potentially any solid tumour commonly treated with chemotherapy agents, would allow determination of the chemosensitivity and best drug combination allowing tailoring of each patient's treatment to their individual tumour response. Furthermore, this could all be achieved within a realistic time frame.

In practice this would mean tissue will be obtained at the time of diagnostic biopsy of a suspected new or recurrent HNSCC and placed in a micro fluidic device. The tissue samples will be cultured and subjected to chemotherapy agents whist the diagnostic biopsy is undergoing formal histological examination and additional radiological imaging is performed to determine nodal and distant metastatic spread. Based on histological, radiological and patient information, the treatment plan is then determined by the multidisciplinary team at 1 to 3 weeks after the diagnostic biopsy. As this thesis has shown that response to chemotherapy can be determined

over a 9-day period, information derived from micro fluidic studies (e.g. chemosensitivity or most cytotoxic regime) can be incorporated into discussions. It is envisaged that a limitation to this approach may be the physical amount of tissue biopsy available to be studied.

As described in section 1.6, radiotherapy is often used as a primary modality (e.g. in early glottic tumours) or as an adjunct to surgery to reduce local recurrent. The relative portability of the micro fluidic device setup will allow tumour biopsies to be irradiated 'on chip'. The cytotoxic effects of radiotherapy can be measured by means of LDH assay on the incubating tumour biopsies. Radiotherapy can also be combined with chemotherapy (incorporating the use of cetuximab for example), allowing for the complete *in vitro* replication of clinical management. Colleagues in the department have commenced these studies that are running in parallel with the chemotherapy experiments.

This thesis has demonstrated the possible applications of micro fluidic technology and although as a 'proof of concept' a real clinical application seems possible, there is one unfulfilled aim. The true correlation with the *in vivo* tumour behaviour is yet to be demonstrated. The key ongoing work from this thesis is therefore to correlate the response to chemotherapy of the individual tumour on chip to the overall clinical response of the patient. This has commenced; to date 6 tumour biopsies have been stored (1 of which was used for section 3.4.4.3) for patients undergoing chemotherapy as part of their treatment for HNSCC. Further work needs to be undertaken to evaluate the response of these tumours to chemotherapy within the micro fluidic device. The clinical response of these patients will be followed for three years for maximal correlation and validation with *in vitro* findings. The continuation of this work, based on the current throughput of the department, would equate to the collection and evaluation of 300 individual patient biopsies over a 3-year period.

Micro fluidic technology has the potential to revolutionise both our understanding of the basic science, and our clinical management of tumours. Its ability to replicate the tumour microenvironment *in vitro* and provide a testing platform provides almost endless opportunities to study tumour behaviour, genetics and pharmacology to name but a few. With further refinement of this technology,

its immediacy and simplicity will allow for ‘personalised chemotherapy at the bedside’ in years to come.

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# Appendix

## I Staging of Head and Neck Sub Sites

### 1.1 Oropharynx

| | |
|---|---|
| T1 | Tumour ≤ 2cm |
| T2 | Tumour >2cm but ≤ 4cm |
| T3 | Tumour >4cm |
| T4a | Invades any of the following: deep muscles of the tongue, larynx, medial pterygoid, mandible and hard palate |
| T4b | Invades any of the following: lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base; or encases the carotid artery |

### 1.2 Nasopharynx

| | |
|---|---|
| T1 | Tumour confined to the nasopharynx |
| T2a | Tumour extends to soft tissues of oropharynx and/or nasal cavity without parapharyngeal extension |
| T2b | Tumour extends to soft tissues of oropharynx and/or nasal cavity with parapharyngeal extension |
| T3 | Tumour invades bony structures and/or paranasal sinuses |
| T4 | Tumours with intracranial extension and/or involvement of cranial nerves, infratemporal fossa, hypopharynx, orbit or masseter space |

### 1.3 Hypopharynx

| | |
|---|---|
| T1 | Tumour limited to one sub site of hypopharynx ≤ 2cm |
| T2 | Tumour invades more than one sub site of hypopharynx or an adjacent site, or measures 2–4 cm, without fixation of hemi larynx |
| T3 | Tumour > 4cm, or with fixation of hemi larynx |
| T4a | Tumour invades any of the following: thyroid/cricoid cartilage, hyoid bone, thyroid gland, oesophagus, central compartment soft tissue |
| T4b | Tumour invades prevertebral fascia, encases carotid artery or invades mediastinal structures |

### 1.4 Larynx

| | |
|---|---|
| T1a | Tumour limited to one vocal cord (may involve anterior or posterior commissure) with normal mobility |
| T1b | Tumour involves both vocal cords (may involve anterior or posterior commissure) with normal mobility |
| T2 | Tumour extends to supraglottis and or subglottis, and/or with impaired vocal cord mobility |
| T3 | Tumour limited to larynx with vocal cord fixation and/or invades paraglottic space, and/or with minor thyroid cartilage erosion (inner cortex) |
| T4a | Tumour invades through thyroid cartilage or invades tissues beyond the larynx, e.g. trachea, soft tissues of neck including deep muscles of tongue, strap muscles, thyroid and oesophagus |
| T4b | Tumour invades prevertebral space, mediastinal structures, or encases carotid artery |

TNM classification of HNSCC, common sites (Sobin et al., 2002)

## II Information Sheets and Consent Forms

UNIVERSITY OF Hull

**Research Participant Information Sheet**

# The use of micro fluidic devices for head and neck cancer biopsies in the evaluation of tumour biology and treatment response, correlated with patient outcome

**Invitation**

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear, or you would like more information.

**Purpose of the study**

This study forms the basis of a Medical Doctorate project.
The University of Hull has developed a method to keep small pieces of tissue functioning for a number of days outside of the body on a micro-chip. We feel that investigating the effects of drug treatments on this tissue can give us better information as to how drug treatments work on tissue in the body, and the potential to develop a system for giving 'personalised chemotherapy'.

**Why have I been chosen?**

We are inviting you to take part in this study as you are undergoing a procedure in which your surgeon may take a biopsy of tissue for diagnostic purposes or as part of your operation. This piece of tissue will be useful to us regardless of your diagnosis.

**Do I have to take part?**

It is up to you to decide. We will describe the study and go through this information sheet, which we will then give you to keep. If you decide to take part we will ask you to sign a consent form, to show you have agreed to take part.

**Am I free to withdraw at anytime?**

You are free to withdraw at any time without giving a reason for your withdrawal. This will not affect the standard of care you receive. Samples already collected will continue to be stored and any information gained from these used in this study.

**What will happen to me if I take part?**

If you agree to take part, an extra sample of tissue will be taken for our study in addition to that taken for routine diagnostic testing. Once your samples are taken they will be only identifiable by a number: non-clinical laboratory staff will not have any access to your personal details. Information regarding your response to treatment and outcome (if applicable) will be accessed by the clinical staff involved with your treatment, and possibly communicated with the research staff. You will not be contacted by the research team after today.

**Expenses and Payment**

You should not incur any expenses during participation. There will not be any financial benefit if this research leads to the development of a new treatment or test.

**What are the possible risks of taking part?**

There are no risks to you or your treatment if you take part.

**What are the benefits of taking part?**
There will be no direct benefit to you for taking part. The information we get from studying your sample will potentially help us with treatments for other patients in the future.

**What do I do if I have a complaint?**
Any complaint about the way you have been dealt with during the study can be addressed by **Professor Stafford, Consultant Surgeon on 01482 465348**. Alternatively you can contact the Patient Advice and Liaison Service (PALS) on 01482 623065.

**Will my taking part in the study be kept confidential?**
Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. Your sample will be identifiable only by a number that can only be linked back to personal details via your clinical care team (i.e. Professor Stafford).

**What will happen to the samples I give?**
The samples will be taken to the research laboratory at Castle Hill Hospital where we will investigate the effect of drug treatments on them. Following completion of the study excess samples will remain stored in liquid nitrogen, securely and anonymously for possible use in future research for a similar project. In this instance further Research Ethical Approval will be sought.

**What will happen to the results of the research study?**
The results of the study maybe published in a scientific journal. At no time will you be identifiable to the reader. No feedback will be given to you unless specifically requested, in which instance the research team will be happy to discuss the general findings.

**Who is organising and funding the research?**
The Head and Neck Cancer research team from the University of Hull will be organising and conducting the research in the laboratories at Castle Hill Hospital. This research is partly funded by the Royal College of Surgeons of England.

**Who has reviewed the study?**
All research in the NHS is looked at by an independent group of people called a Research Ethics Committee to protect your interests. This study has been reviewed and given favourable opinion by the Hull and East Riding Local Research Ethics Committee.

**Further information**
If you require any further general or specific information on the research, advice on whether to take part, or if you are at all unhappy please contact Professor Stafford on 01482 465348.
Thank you for taking the time to read this information sheet.

**Consent**
If you decide to take part in the study, we will provide you with a copy of the information sheet and ask you to sign a study Consent Form.

UNIVERSITY OF Hull

**CONSENT FORM**

Centre Number:
Study Number:
Patient Identification Number for this trial:

## The use of micro fluidic devices for head and neck cancer biopsies in the evaluation of tumour biology and treatment response, correlated with patient outcome

1. I confirm that I have read and understand the information sheet dated 14.1.10 (version 2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason, without my medical care or legal rights being affected. If I do withdraw, samples already obtained may still be used. ☐

3. I understand that I am under no obligation to take part and that, if I agree to take part, I am free to change my mind at any time. ☐

4. I understand that I will not benefit financially if this research leads to the development of a new treatment or test. ☐

5. I give permission for an additional sample of tissue to be taken during my operation to be used for this research. ☐

6. I agree that any samples not used in this study may be stored and used in future research of a similar nature pending ethical approval. ☐

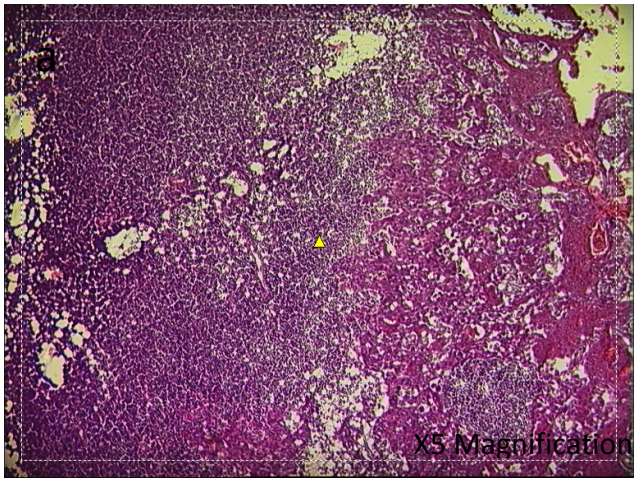
7. I understand that these samples will be stored anonymously and I consent to the information derived from them to be analysed by computer. ☐

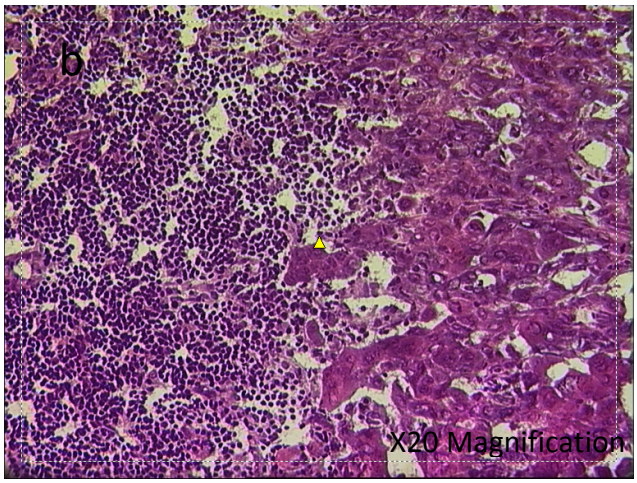
8. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the University of Hull, from regulatory authorities, or from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

9. I hereby freely give my consent to take part in this study.

| _____ | _____ | _____ |
|---|---|---|
| Name of Volunteer | Date | Signature |
| _____ | _____ | _____ |
| Name of Person taking consent | Date | Signature |

## III Additional Histology Figures



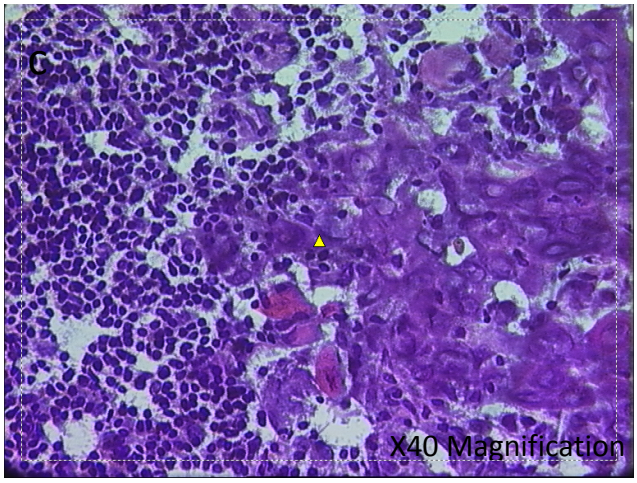
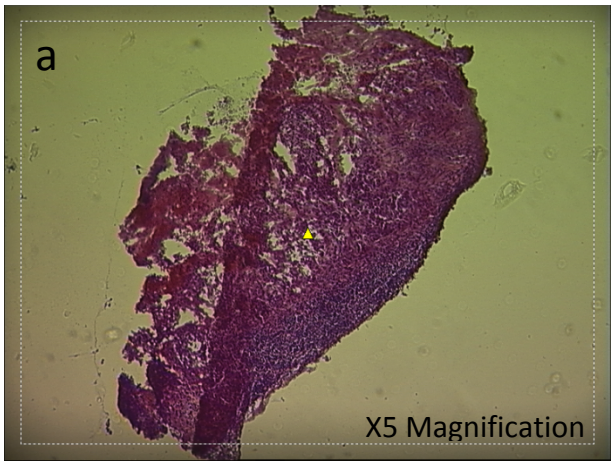
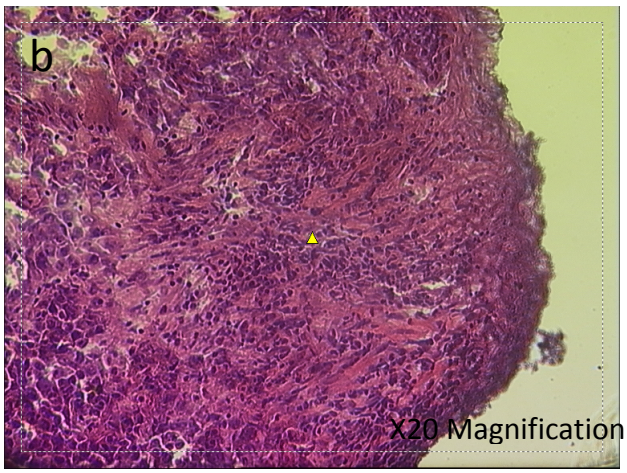




Figure 1 Image of tumour/node prior to incubation from tumour biopsy edge at a) x5, b) x20, c) x40 magnification. Areas of SCC (left side) meeting areas of nodal tissue (right side).



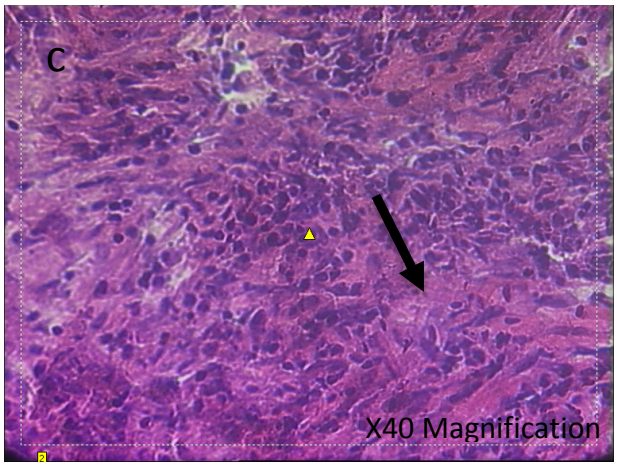
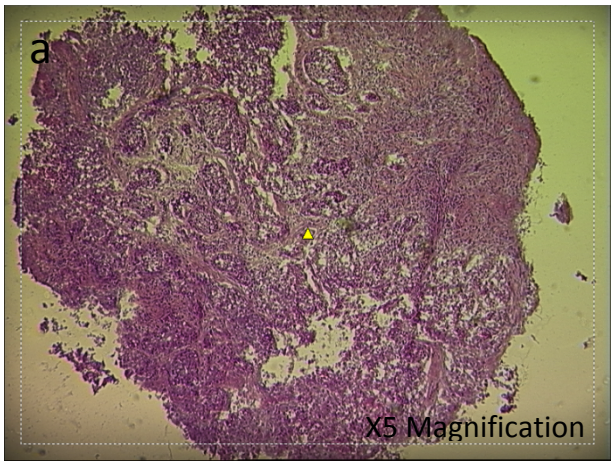
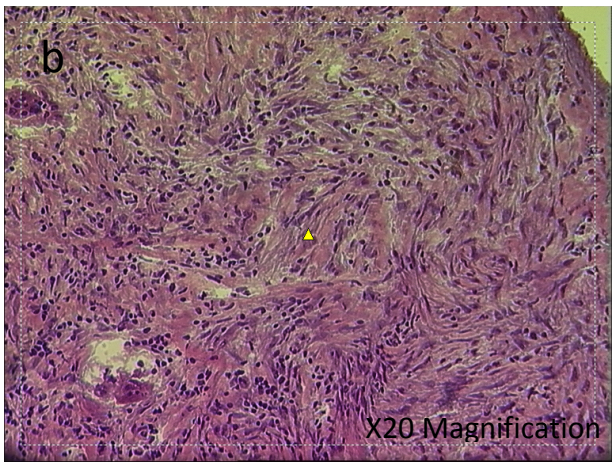




Figure 2 Image of yumour/node at 48 h incubation from tumour biopsy edge at a) x5, b) x20, c) x40 magnification. Areas of cell viability; rounded nuclei and maintained cell cohesion at edge and centre of tissue. Some areas degraded nuclei (arrow).



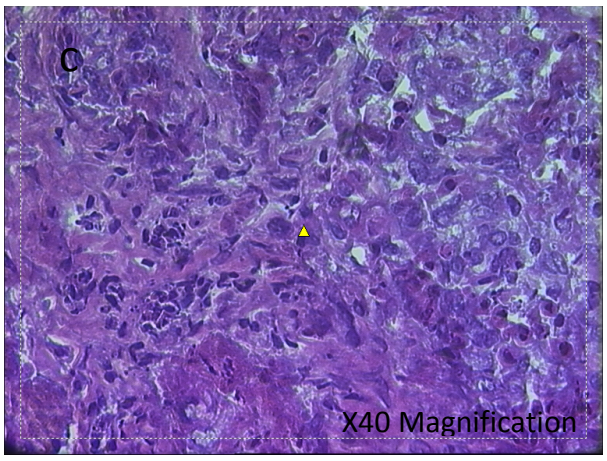




Figure 3 Image of tumour/node at 96 h incubation from intermediate area of tumour biopsy at a) x5, b) x20, c) x40 magnification. Areas of cell viability will cohesion maintained.  Some nuclei degradation but some intact nuclei identifiable.