The Detection of Circulating Tumour Cells in Oesophageal Adenocarcinoma

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Abstract

The incidence of oesophageal adenocarcinoma is rising. Overall survival rates remain poor. Traditional methods of staging oesophageal adenocarcinoma fail to identify patients at high risk of early disease recurrence after surgical treatment with curative intent. Circulating tumour cells have been reported to offer prognostic information in patients with certain tumour types including breast, prostate and colorectal. The majority of studies have focused on circulating tumour cell enumeration in patients with metastatic disease. Little is known about the role of circulating tumour cells in oesophageal adenocarcinoma or of the importance of circulating tumour cells in patients without metastatic disease.

Cultured oesophageal adenocarcinoma cell lines were used to develop and validate a novel method for detection of tumour cells in whole blood. The method used positive depletion of normal blood cell populations before imaging the cells with an Imagestream^{x} image flow cytometer. A panel of fluorescently-conjugated antibodies against EpCAM, cytokeratins, survivin, CD45 and DAPI were used to discriminate the tumour cells.

A consistent recovery of 48% was achieved across a range of concentrations of cultured oesophageal adenocarcinoma cells added to whole blood. Blood samples from 25 patients undergoing staging for oesophageal adenocarcinoma without known distant metastatic disease were studied. Circulating tumour cells were identified in 5 patients, range 2 – 85 per 5 ml whole blood. Circulating tumour-associated macrophages were identified in a single patient. No difference in overall survival was demonstrated between those patients with circulating tumour cells compared to those without.

The developed method produces high quality images allowing for the detection and characterisation of circulating tumour cells. Heterogeneity within the circulating tumour cell population was observed. Circulating tumour cells may be identified in a significant number of patients with oesophageal adenocarcinoma without radiological evidence of distant metastatic disease.

Table of Contents

List of Figures ix

List of Tables xii

Chapter 1. Introduction 1

1.1 Oesophageal Cancer 1

1.1.1 Symptoms 2

1.1.2 Diagnosis 3

1.1.2 Histological subtypes 3

1.1.4 Staging 6

1.1.5 Junctional Tumours 11

1.1.6 Anatomical Considerations 12

1.1.7 Treatment 14

1.1.8 Non-surgical Treatments 17

1.1.9 Outcomes 18

1.1.10 The Northern Oesophago-Gastric Cancer Unit 18

1.1.11 Disease recurrence 19

1.2 Circulating Tumour Cells 20

1.2.1 Methods of circulating tumour cell detection 22

1.2.2 CellSearch® (Veridex) 23

1.2.3 Imagestream^{x} (Amnis) 25

1.2.4 Reverse transcriptase-PCR 26

1.3 Biomarker Selection 27

1.3.1 EpCAM 27

1.3.2 Epithelial mesenchymal transition 28

1.3.2 Cytokeratins 29

1.3.3 Survivin 30

1.4 Clinical Value of Circulating tumour cells 31

1.4.1 CTCs in Oesophageal Cancer 32

1.4.2 The potential role for circulating tumour cells in the management of oesophageal adenocarcinoma 35

1.5 Aims and objectives 38

Chapter 2. Materials and methods 39

2.1 Commercial cancer cell lines 39

2.2 Tissue Culture 40

2.3 Immunofluorescence 40

2.4 Image flow cytometry (Imagestream^{x}) 41

2.5 Compensation Matrix 42

2.6 Antibody labelling of samples for image flow cytometry 43

2.7 Analysis of patient samples 43

2.8 Recovery of cancer cells form whole blood 44

2.9 EasySep™ 45

2.10 Final protocol for analysing patient samples 46

2.11 Statistical Analysis 47

Chapter 3. The use of image flow cytometry to detect and characterise oesophageal adenocarcinoma cells 49

3.1 Introduction 49

3.2 Results 49

3.2.1 Image acquisition and quality using Imagestream^{x} 49

3.2.2 Image Compensation 52

3.2.3 Image selection using image flow cytometry 56

3.2.4 Quantification of fluorescence 59

3.2.5 The effect of masks on quantification of fluorescence 60

3.2.6 EPCAM expression in oesophageal cancer cell lines 63

3.2.7 Cytokeratin expression in oesophageal cancer cell lines 66

3.2.8 Survivin expression in oesophageal cancer cell lines 69

3.3 Discussion 71

Chapter 4. Development of a method to enrich circulating tumour cells from whole blood 74

4.1 Introduction 74

4.2 Results 76

4.2.1 Morphology and cross-sectional area of cancer cells in comparison to white blood cells 76

4.2.2 Panel of antibodies for cancer cell detection 78

4.2.3 White blood cell depletion 79

4.2.4 Imagestream^{x} IDEAS® software enrichment 81

4.2.5 Losses associated with the Imagestream^{x} image flow cytometer 84

4.2.6 Recovery of oesophageal adenocarcinoma cells from whole blood 86

4.2.7 Healthy Volunteer Samples 87

4.3 Discussion 87

Chapter 5. Patient samples 92

5.1 Introduction 92

5.2 Results 92

5.2.1 Patient Demographics 92

5.2.2 Circulating tumour cell heterogeneity 94

5.2.3 Circulating tumour cell area 98

5.2.4 Additional objects identified from patient samples 100

5.2.5 Patient Outcomes 104

5.3 Discussion 107

Chapter 6. Concluding Discussion 108

6.1 Summary of work 108

6.2 Patient cohort 108

6.3 Cohort size 110

6.4 Biomarker selection 110

6.5 Host response 111

6.6 Limitations of circulating tumour cell research 112

References 115

Appendix A. Patient consent form 132

Appendix B. Manuscript 137

List of Figures

Figure 1.1 The average number of new cases of oesophageal cancer per year in the UK and the age-specific incidence rates between 2009-2011. 2

Figure 1.2. Examples of oesophageal adenocarcinoma detected at oesophago-gastro-duodenoscopy. 3

Figure 1.3. Endoscopic appearance of Barrett's oesophagus. 5

Figure 1.4. The UICC TNM staging classification (7th edition) for oesophageal adenocarcinoma. 8

Figure 1.5. Staging pathway for patients diagnosed with oesophageal adenocarcinoma. 10

Figure 1.6. The extent of nodal dissection performed during oesophagectomy. 13

Figure 1.7. Subtotal oesophagectomy 15

Figure 1.8. Funnel plot of the number of resections performed and 30 day mortality rates for all UK oesophago-gastric surgical units. 19

Figure 1.9. Schematic representation of the development of circulating tumour cells. 20

Figure 1.10. Examples of circulating tumour cell images captured using the Cellsearch® platform from blood samples of cancer patients. 24

Figure 2.1. Oesophageal adenocarcinoma cell line in culture. 40

Figure 2.2 Imagestream^{x} Mark II image flow cytometer. 42

Figure 2.3. Depletion of white blood cells with the EasySep™ 46

Figure 3.1 Comparison of images captured by traditional immunofluorescence and Imagestream^{x} image flow cytometry. 52

Figure 3.2. Common fluorophores and the corresponding excitation lasers and collection channels for the Imagestream^{x} image flow cytometer 53

Figure 3.3. The effect of compensation on image intensity using image flow cytometry 56

Figure 3.4. Selection of single cell images using image flow cytometry 59

Figure 3.5. Methods of quantification of fluorescence captured by image flow cytometry.....60

Figure 3.6. The effect of masks on quantification of image flow cytometry fluorescence.....62

Figure 3.7. The limitation of use of a two dimensional mask when applied to a three dimensional cell.....63

Figure 3.8. EpCAM expression across oesophageal adenocarcinoma cell lines 66

Figure 3.9. Cytokeratin expression in oesophageal adenocarcinoma cell lines 69

Figure 3.10. Survivin expression in oesophageal adenocarcinoma cell lines.....71

Figure 3.11. Published images of circulating tumour cells detected and imaged using the Cellsearch® platform 72

Figure 4.1. Morphology and cross-sectional cell and nuclear areas of cancer cells in comparison to white blood cells 78

Figure 4.2. Detection of oesophageal adenocarcinoma cells in whole blood by image flow cytometry 79

Figure 4.3. Depletion of white blood cells by EasySep™ CD45 depletion.....81

Figure 4.4. Discrimination of the malignant cell population in whole blood from the residual leukocytes after positive depletion of blood cells. 83

Figure 4.5. The effect of sample volume on sample loss during image flow cytometry. 85

Figure 4.6. Recovery of cultured oesophageal adenocarcinoma cells from whole blood 87

Figure 5.1 Comparison of individual circulating tumour cells from a single patient (patient 006).....95

Figure 5.2 Circulating tumour cells detected in patients with oesophageal adenocarcinoma 98

Figure 5.3 Size of circulating tumour cells detected in patient samples compared with oesophageal adenocarcinoma cell lines 99

Figure 5.4 Fluorescently-labelled white blood cell population identified during processing of patient samples 101

Figure 5.5 Fluorescently-labelled debris identified during processing of patient samples 102

Figure 5.6 Circulating tumour-associated macrophages identified in patient samples 104

Figure 5.7 Survival of patients included in study 106

List of Tables

Table 1. Risk factors for the two common histological subtypes of oesophageal cancer. 4

Table 1.2. Siewert and Stein classification of tumours of the gastro-oesophageal junction....12

Table 1.3. The types and numbers of different types of cells in human blood....22

Table 2.1. Histology and origin of selected oesophageal adenocarcinoma cell lines. 39

Table 4.1 Theoretical losses of recovery at sequential stages of the enrichment and labelling of circulating tumour cells. 89

Table 5.1 Patient demographic and staging information....94

Table 5.2 Circulating tumour cell numbers and outcomes for patients included in the study....105

Chapter 1. Introduction

1.1 Oesophageal Cancer

Oesophageal cancer is the eighth most common cancer worldwide. In the United Kingdom, it is the thirteenth most common cancer and accounts for three per cent of all cancer cases. The incidence in males is approximately double that in females; it is the eighth most common cancer in males in the United Kingdom. It affects most commonly people over the age of 60 (Figure 1.). The overall prognosis for patients with the disease remains very poor. The five year survival for all patients diagnosed in the United Kingdom is only 15%. This low survival rate is because approximately two thirds of patients have advanced and incurable disease at the time of presentation and diagnosis. Clearly, novel approaches to the earlier diagnosis and treatment of oesophageal cancer are required.

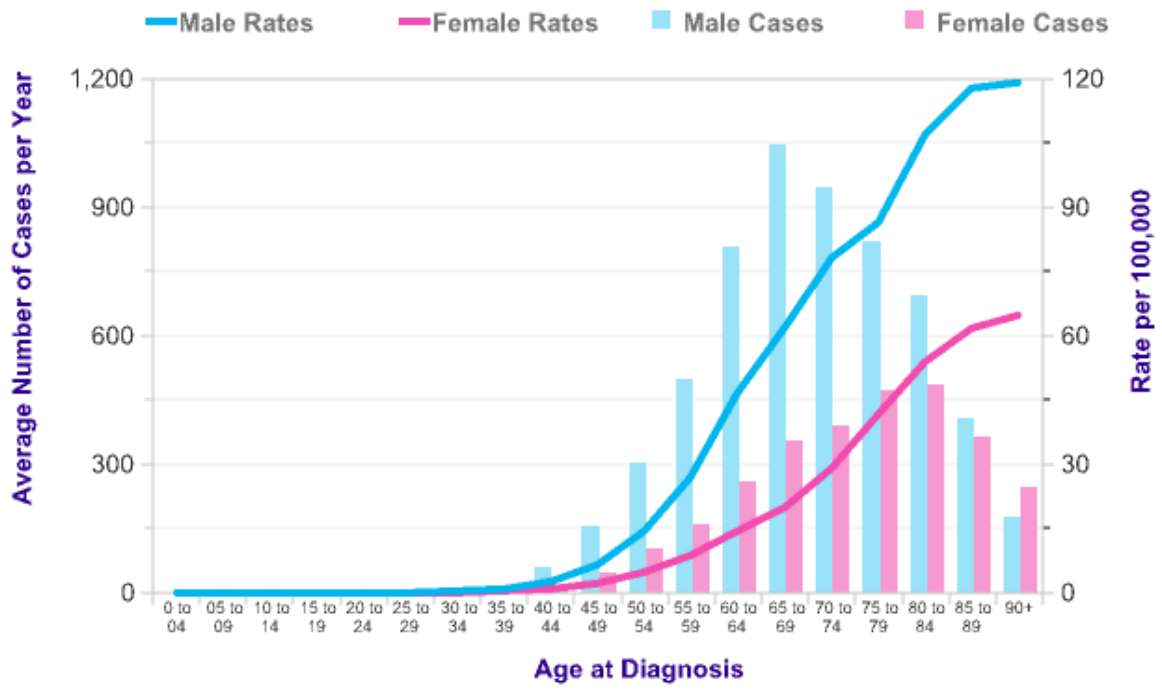


Figure 1.1 The average number of new cases of oesophageal cancer per year in the UK and the age-specific incidence rates between 2009-2011.

The incidence of oesophageal cancer per 100,000 males remains nearly double that for females although the total number of cases in both sexes gradually converges with increasing age due to differences in overall life expectancy. Figure reproduced from (Cancer Research UK Oesophageal Cancer Incidence Statistics)

1.1.1 Symptoms

The commonly described symptoms of oesophageal cancer include dysphagia and weight loss. These symptoms are usually associated with advanced disease (Bowrey *et al.*, 2006). More rapid investigation of patients who present to their general practitioner with these symptoms has been shown to reduce the time to diagnosis but has no benefit on survival (Sharpe *et al.*, 2010). In early stages, oesophageal cancer may be asymptomatic or present with more subtle symptoms. The links between heartburn, Barrett's metaplasia and oesophageal adenocarcinoma are well recognised (Lagergren *et al.*, 1999). Heartburn and reflux are therefore potential early symptoms of oesophageal adenocarcinoma, although they are more commonly associated with non-malignant pathology (Rubenstein *et al.*, 2011). Recent awareness campaigns, including the national 'Be Clear on Cancer' campaign in

2015, have focused on the importance of individuals reporting their persistent heartburn to allow for earlier investigation and an earlier diagnosis of oesophageal cancer to be made.

1.1.2 Diagnosis

The gold standard investigation for patients suspected of having oesophageal cancer is flexible oesophago-gastro-duodenoscopy (Adams *et al.*). This procedure allows visual assessment of any tumour and permits biopsies to be taken for histological confirmation (Figure 1.2). A small proportion of patients have their tumour identified first by computed tomography scan (Bettegowda *et al.*) or by contrast barium swallow.

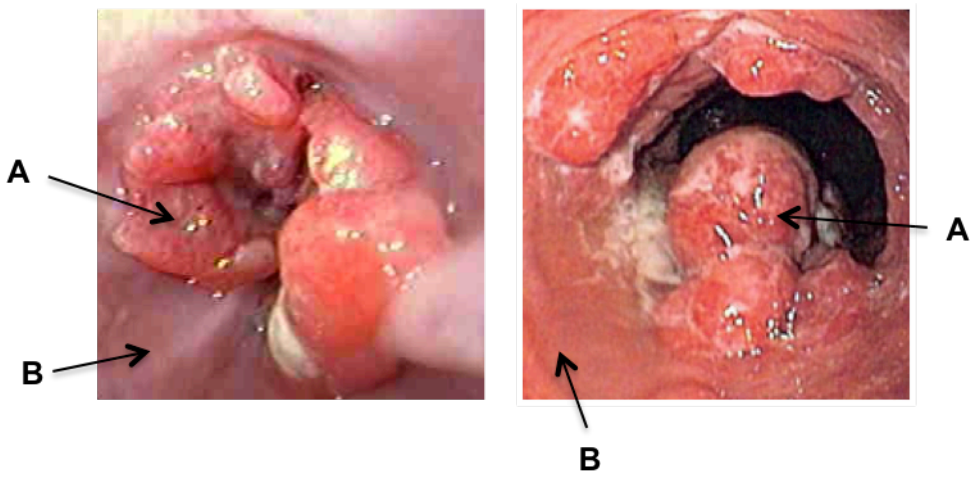


Figure 1.2. Examples of oesophageal adenocarcinoma detected at oesophago-gastro-duodenoscopy.

In both images an irregular, thickened tumour (A) is easily distinguished from the normal smooth squamous mucosa (B) of the distal oesophagus.

1.1.2 Histological subtypes

There are two common histological subtypes of oesophageal cancer; adenocarcinoma and squamous cell carcinoma. Rarer subtypes include small cell cancer and neuroendocrine tumours. Squamous cell carcinomas arise from the

native squamous mucosa of the oesophagus and may occur throughout the oesophagus. By contrast adenocarcinomas usually arise within the distal third of the oesophagus. The incidence of the two subtypes demonstrates considerable geographical variation. In Eastern countries, virtually all oesophageal cancer is squamous cell carcinoma. By contrast in the West, although both subtypes of cancer are encountered, adenocarcinoma is more prevalent. The incidence of oesophageal squamous cell carcinoma in the West has remained relatively stable but incidence rates of oesophageal adenocarcinoma continue to rise (Vizcaino *et al.*, 2002). The highest reported incidence of oesophageal adenocarcinoma is in the United Kingdom (Bollschweiler *et al.*, 2001).

Histological subtype	Risk Factors
Squamous cell carcinoma	• Smoking • Alcohol • Hot drinks • Family history • Riboflavin deficiency • Vitamin A and C deficiency • Corrosive strictures • Achalasia • Tylosis palmarum
Adenocarcinoma	• Gastro-oesophageal reflux (Hahn *et al.*) • Barrett's metaplasia of the oesophagus • Obesity

Table 1. Risk factors for the two common histological subtypes of oesophageal cancer.

The aetiology and risk factors differ considerably for the two common histological subtypes of oesophageal cancer as shown in Table 1.1. Oesophageal adenocarcinoma is most commonly caused by gastro-oesophageal reflux (Lagergren *et al.*, 1999; Rubenstein and Taylor, 2010). Risk factors for oesophageal adenocarcinoma therefore include factors that increase the risk of acid reflux. Acid reflux can lead to a cellular change to the normal lining of the oesophagus. The stratified squamous mucosa undergoes a process of glandular metaplasia. This metaplasia is known as Barrett's metaplasia of the oesophagus (figure 1.3). It is

estimated that Barrett's metaplasia of the oesophagus affects up to 2% of the UK population (Jankowski *et al.*, 2010). Within the Barrett's mucosa, dysplastic changes may occur to the epithelial cells. These changes are defined as low or high grade dysplasia and when identified have a high rate of progression to the development of invasive adenocarcinoma. This progression rate is estimated to be between 0.22-0.26% per year for patients with known Barrett's oesophagus (Bhat *et al.*, 2011; Hvid-Jensen *et al.*, 2011). The progression from acid reflux, Barrett's metaplasia of the oesophagus and subsequent development of oesophageal adenocarcinoma is therefore well established (Lagergren *et al.*, 1999; Rubenstein and Taylor, 2010). The increasing rates of obesity amongst the UK and other Western populations have contributed to the increased incidence of oesophageal adenocarcinoma.

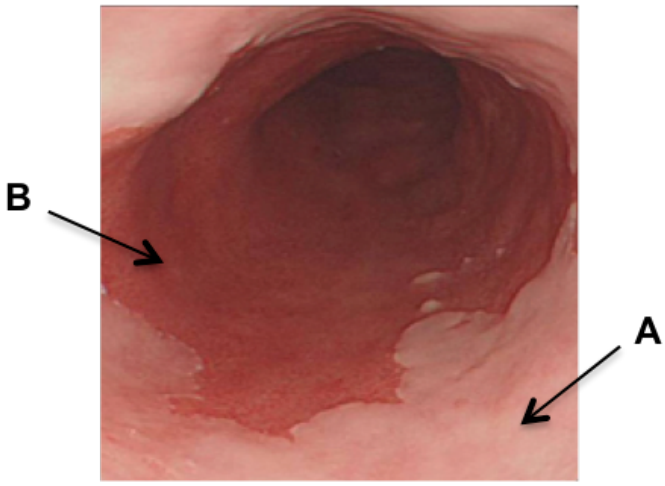


Figure 1.3. Endoscopic appearance of Barrett's oesophagus.

A clear distinction is observed between the pale pink normal oesophageal squamous mucosa (A) and the darker abnormal Barrett's mucosa (B) in the lower third of the oesophagus during flexible oesophago-gastro-duodensoscopy (Adams *et al.*).

Oesophageal adenocarcinoma and squamous cell carcinoma are recognised to be very different diseases in terms of pathogenesis, tumour biology and prognosis (Siewert and Ott, 2007). In the past, both histological types of oesophageal cancer have been studied together and reported in combined series which means that it is not possible to compare and interpret results for the two histological types. It is now recommended that squamous cell carcinoma and adenocarcinoma should be reported separately. Because of the higher incidence of oesophageal

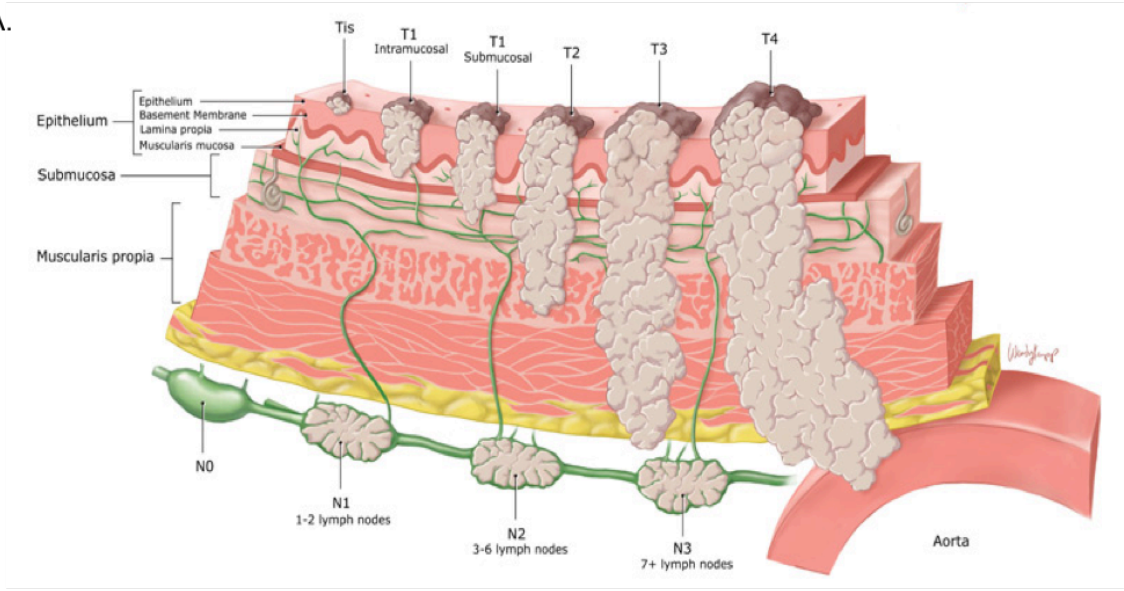
adenocarcinoma within the UK and its rapid increase, this study has focused on oesophageal adenocarcinoma alone.

1.1.4 Staging

Staging describes the extent of a patient's tumour both in terms of local growth of the primary tumour and dissemination to lymph nodes or other metastatic sites. It separates patients with the disease into prognostic groups upon which treatment decisions can be based. Oesophageal adenocarcinoma is staged according to the Union for International Cancer Control (UICC) TNM classification. The TNM staging system was devised for all solid tumours by Pierre Denoix and is now maintained by the UICC. This classification provides information on the primary tumour (T), lymph nodes (N) and distant metastatic (M) disease. The most up-to-date version of the TNM classification for oesophageal adenocarcinoma is the 7th Edition. (Figure 1.4A) (Rice *et al.*, 2010). The primary tumour is classified from T1 to T4 depending on the depth of invasion through the oesophageal wall. T4 disease represents local invasion into adjacent structures. Tis refers to carcinoma in-situ and is regarded as a pre-malignant condition which lacks the characteristic invasion seen in malignancy. It is more commonly referred to as high grade dysplasia. Nodal disease is classified from N1 to N3 according to the number of nodes that are involved with metastatic tumour deposits. Finally, distant metastatic disease is classed as M1 with M0 used to describe the absence of such disease spread. A tumour is therefore described, for example, as T3N1M0.

Individual tumour stages are further classified into stage groups as detailed in figure 1.4B. Stage groups provide prognostic information; a more advanced stage group has a worse survival. Stage four represents distant metastatic disease. Common sites for metastatic dissemination of oesophageal adenocarcinoma include the liver, lungs and bone. Involvement of lymph nodes that are very distant from the tumour, for example in the neck, are also classified as metastatic (M1) disease.

A.



B.

	Adenocarcinoma			
Stage	T	N	M	Grade
0	Is	0	0	1
1A	1	0	0	1-2
1B	1 2	0	0	3 1-2
2A	2	0	0	3
2B	3 1-2	0 1	0	Any
3A	1-2 3 4a	2 1 0	0	Any
3B	3	2	0	Any
3C	4a 4b Any	1-2 Any 3	0	Any
4	Any	Any	1	Any

Figure legend on next page

Figure 1.4. The UICC TNM staging classification (7th edition) for oesophageal adenocarcinoma.

A. Schematic of the TNM classification. The multiple layers of the oesophageal wall are illustrated. T stage (Tis-T4) is determined by the depth of invasion of the primary tumour. Tis represents in-situ disease in which the tumour has Nodal stage (N0-N3) reflects the number lymph nodes involved with tumour. Distant metastatic disease is classified as M0 (No metastatic disease) or M1 (metastatic disease present). Figure reproduced from (University Hospitals Cleveland, USA) B. Stage groups according to the UICC TNM classification. Individual TNM stages are grouped together according to prognostic information.

Staging is essential to allow appropriate treatments to be planned for patients (see section 1.1.7). Patients with stage 4 disease have incurable disease and are only suitable for palliative therapies. Locally advanced (T4) oesophageal adenocarcinoma describes tumours that have directly invaded adjacent structures for example the aorta or lung and hence may not be suitable for curative treatment. For patients who are suitable for treatment with curative intent, staging informs the selection of appropriate treatment modalities, for example peri-operative chemotherapy and surgery. The most commonly used peri-operative chemotherapy is only offered to patients with either T3 or node positive (N≥1) disease (Cunningham *et al.*, 2006). Definitive TNM staging requires histological examination of the resection specimen.

Current pre-treatment staging of patients includes radiological assessment of the disease. The radiological staging protocol for oesophageal adenocarcinoma is more complicated than for most other tumour types because of the anatomical location of the oesophagus, its proximity to other structures such as the heart, lungs and aorta, and the complex lymphatic drainage from the oesophagus into both the thorax and abdomen. A summary of the staging pathway for oesophageal cancer is provided in Figure 1.5. All patients undergo a computed tomography (CT) scan (Bettegowda *et al.*) that provides information about the size and position of the primary tumour, the involvement of local lymph nodes and the presence of distant metastatic disease. A significant proportion of patients will be found to have incurable disease by CT scan alone and will undergo no further staging tests. Those patients whose disease is classed as curable by CT scan will frequently undergo both endoscopic ultrasound (EUS) and positron emission tomography-computed tomography (PET-CT). Endoscopic ultrasound involves an ultrasound probe attached to the end of a gastroscope that allows accurate determination of the depth of tumour invasion (T

stage) and local nodal involvement. PET-CT utilises the increased metabolism of glucose by tumour cells compared with native tissues. Fluorodeoxyglucose (^{18}F-FDG), a radiolabelled glucose analogue, is injected intravenously and is detected within tumour sites by the positron emission tomography scan. This scan is combined with a traditional CT scan to generate the combined PET-CT images. PET-CT may identify low volume distant metastatic disease that is not easily identified by conventional CT scanning. Patients with lymph nodes within the chest may also undergo an external neck ultrasound to identify involved lymph nodes in the neck. These nodes are outside the surgical field and cannot be removed at surgery. A small number of patients require a staging laparoscopy if the tumour crosses the gastro-oesophageal junction into the proximal stomach. This laparoscopy involves inserting a camera inside the abdominal cavity under general anaesthesia and investigates peritoneal spread of the tumour, the presence of low volume ascites and if the tumour is invading adjacent structures such as the diaphragm.

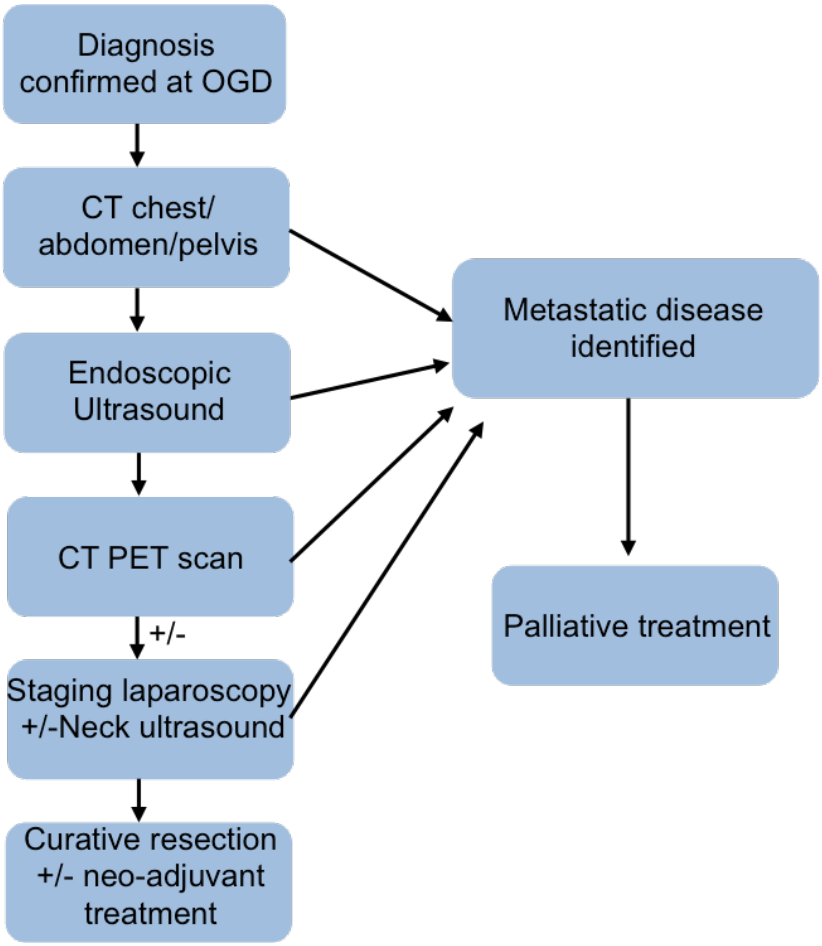


Figure 1.5. Staging pathway for patients diagnosed with oesophageal adenocarcinoma.

All patients undergo a CT scan. Subsequent tests are performed sequentially. The identification of metastatic disease at any stage will stop the pathway as patients are no longer eligible to treatment with curative intent.

What is common to all of these staging modalities is that they are able to detect macroscopic disease. There are limitations to the accuracy of all radiological tests and in particular, low volume metastases are difficult to identify and characterise. At present, the assessment of microscopic metastases does not form part of the pre-treatment staging process for oesophageal adenocarcinoma.

Alongside the staging pathway, a careful assessment of a patient's fitness is required. This assessment is an essential part of planning treatment options. Patients undergo rigorous testing using formal Cardio-Pulmonary Exercise Testing (CPX). Numerous physiological parameters including anaerobic threshold and V02 max are calculated based upon controlled exercise on a static bike. CPX helps to assess and stratify an individual's ability to tolerate surgical resection, however the precise value

of CPX testing in predicting cardiopulmonary complications following oesophagectomy is unclear (Forshaw *et al.*, 2008; Moyes *et al.*, 2013).

1.1.5 Junctional Tumours

The classification of tumours that arise within five centimetres of the gastro-oesophageal junction is complex due in part to a lack of a consensus definition of the gastro-oesophageal junction. The junction may be defined anatomically or based upon the site of change in the mucosa from squamous to columnar. The junction is frequently obscured by tumour which makes identification difficult. Junctional tumours are almost invariably adenocarcinomas but there is considerable debate as to whether they should be managed as oesophageal or gastric tumours. The most widely accepted definition was first described by Siewert and Stein (Siewert and Stein, 1998). This classification describes three types of junctional tumour based upon the anatomical location of the centre of the tumour (Table 1.2). For the purposes of clinical management, type 1 tumours are considered to be oesophageal cancers and type 3 gastric. The management of type 2 lesions is more complicated. Both total gastrectomy via an abdominal approach and trans-thoracic oesophagectomy have been proposed as the surgery of choice for this patient group, reflecting the ability of cells from these tumours to metastasise to lymph nodes in both the abdomen and thorax (Mariette *et al.*, 2011). It is important that all three types of junctional tumours are not grouped with oesophageal adenocarcinomas for the purposes of reporting. Only true oesophageal adenocarcinomas or type 1 junctional tumours are included in this study.

	Description
Type 1	Adenocarcinoma of distal oesophagus which infiltrates the gastro-oesophageal junction from above. The centre of the tumour is within 1-5 cm above the cardia
Type 2	True carcinoma of the cardia arising from gastric cardia epithelium or short segments with intestinal metaplasia at the gastro-oesophageal junction. The centre of the tumour is within 1 cm above and 2 cm below the cardia.
Type 3	Gastric carcinomas that infiltrate the gastro-oesophageal junction from below. The centre of the tumour is within 2-5 cm below the cardia

Table 1.2. Siewert and Stein classification of tumours of the gastro-oesophageal junction

1.1.6 Anatomical Considerations

The oesophagus consists of a cervical, thoracic and abdominal component. The average distance from the teeth to the gastro-oesophageal junction is 40 cm. It is composed of several layers as illustrated in figure 4A. Unlike the majority of the gastro-intestinal tract there is no outer serosal layer. The blood supply and lymphatic drainage is complex. The lower third of the oesophagus is a site of communication between the portal and systemic circulations. Arterial blood supply to the upper third is derived from the inferior thyroid artery, to the middle third direct from the aorta, and to the lower third from the left gastric artery. Venous drainage is equally divided between the inferior thyroid vein, azygos vein and left gastric vein. Lymphatic drainage is equally complex. Lymph drains to nodal groups within both the chest and abdomen. The nodal groups are described in tiers as illustrated in Figure 1.6. This varied blood supply and lymphatic drainage means that there is considerable variation in the ways in which tumour cells metastasise in oesophageal adenocarcinoma. Lymph node involvement in particular is possible in both the chest and abdomen (Dresner *et al.*, 2001). The treatment of oesophageal cancer with either surgery or radiotherapy must therefore take into account the impact of this complex anatomy.

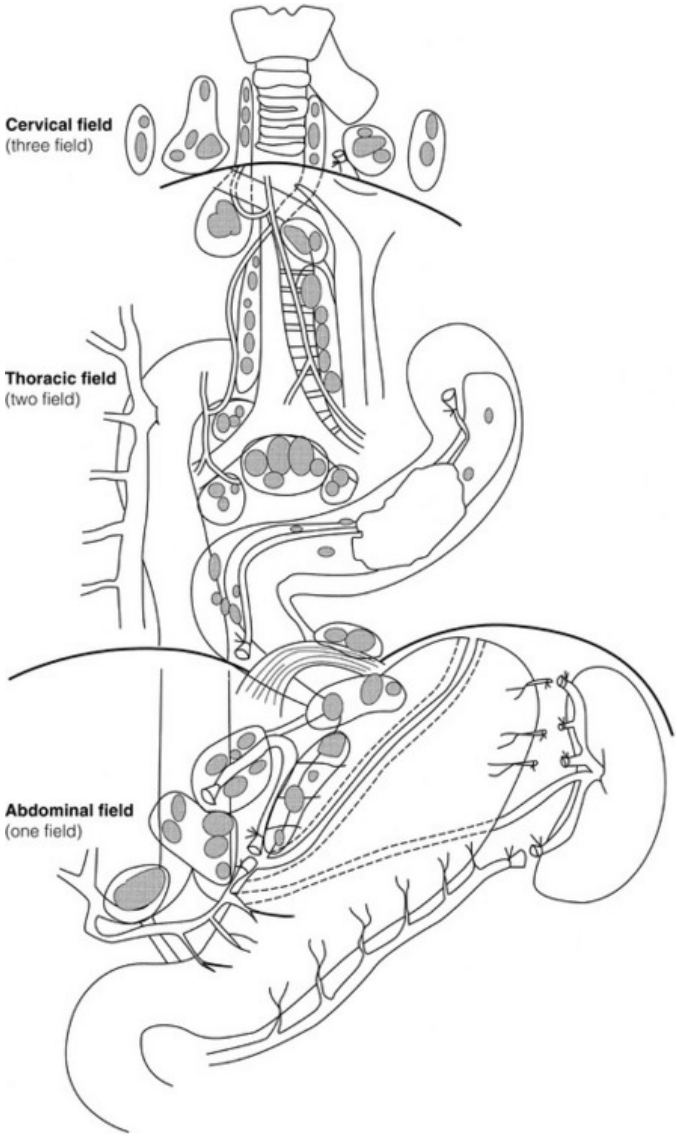


Figure 1.6. The extent of nodal dissection performed during oesophagectomy.

The nodal locations are displayed anatomically and divided into three fields of dissection. A standard subtotal oesophagectomy is performed with en-bloc dissection of the abdominal and thoracic fields (two field dissection). Cervical field nodes are removed only with more proximal oesophageal cancers, the majority of which are squamous cell carcinomas. Reproduced from (Griffin SM et al., 2013)

1.1.7 Treatment

Surgery

The mainstay of curative treatment for patients with oesophageal adenocarcinoma is surgical resection which is called oesophagectomy. The most common procedure is a two stage oesophagectomy with two field lymphadenectomy. Briefly, the stomach is mobilised via an abdominal incision and draining lymph node groups are resected. The right gastric and right gastro-epiploic vessels are preserved to ensure an adequate blood supply to the stomach. The oesophagus and surrounding tissue and nodes are mobilised via a right thoracotomy and the mobilised stomach is pulled up into the chest. The distal oesophagus and upper stomach is resected (Figure 1.7A). The residual stomach is anastomosed to the proximal oesophagus to restore gastrointestinal continuity (Figure 1.7B).

There is potential for the manipulation of a tumour during surgery to lead to tumour cell dissemination. Such concerns were first raised by Turnbull in 1967 in colorectal cancer and led to the development of a no touch surgical technique in which lymphovascular pedicles were divided before the tumour was handled. The no touch approach led to improved survival of colorectal cancer patients (Turnbull *et al.*, 1967). In view of the nature of the oesophageal blood and lymphatic supply, such an approach to oesophagectomy is not possible. Tumour handling is kept to a minimum but cannot be avoided entirely.

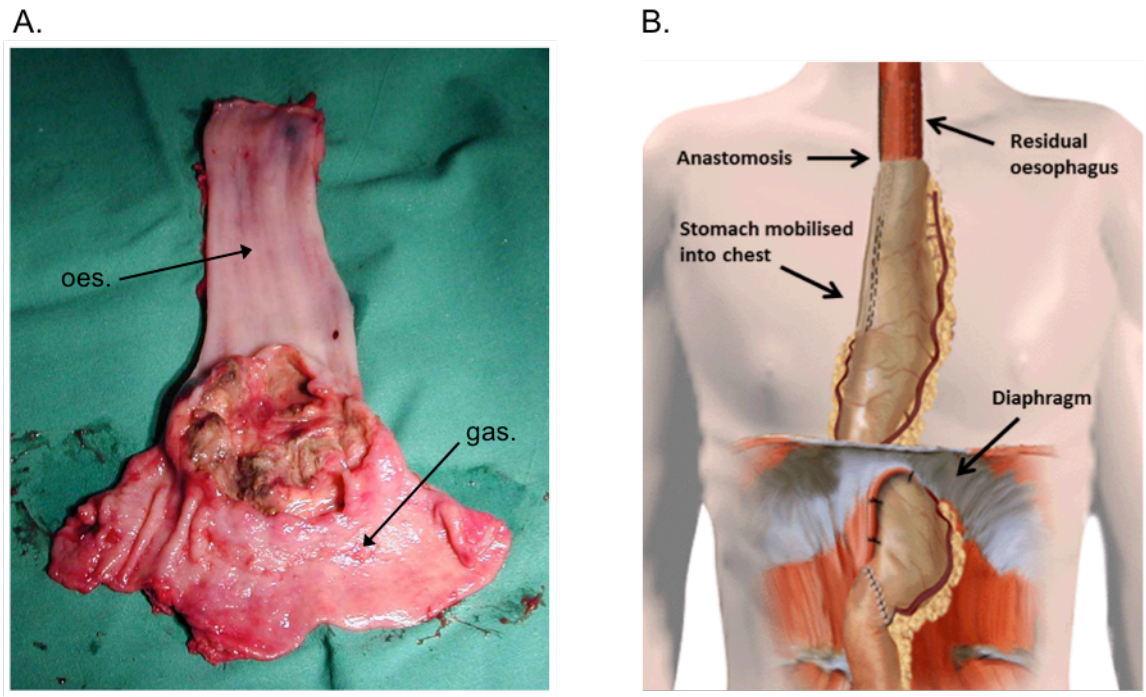


Figure 1.7. Subtotal oesophagectomy

A. Surgical resection specimen following oesophagectomy for oesophageal adenocarcinoma. The specimen has been opened to allow visualisation of the tumour. The oesophageal mucosa (oes.) above the tumour appears normal as does the gastric mucosa below the tumour (gas.). B. Diagram of the major anatomical consequences of oesophagectomy. The mobilised stomach can be seen anastomosed to the residual oesophagus in the thorax to restore continuity of the gastrointestinal tract.

Oesophagectomy represents very major surgery and a major physiological insult to the patient. Historically oesophagectomy was associated with a very high rate of post-operative complications. Patients were in hospital for approximately three weeks and between 20 and 30% of patients died during the post-operative hospital stay. In modern, specialist high-volume centres mortality rates of around 2% are achieved now with lengths of stay in hospital as low as seven days. The rate of morbidity following oesophagectomy remains fairly high however with up to 20% of patients experiencing a significant complication in the immediate post-operative period. As mentioned in section 5.1.4, all patients undergoing surgery will have undergone CPX testing. CPX has been reported to be predictive of complications in patients undergoing an oesophagectomy (Onat *et al.*).

Improvements in post-operative survival have led to a much better understanding of the impact of oesophagectomy on quality of life. Patients take on average between nine and twelve months to recover their baseline quality of life following oesophagectomy (Jacobs *et al.*, 2014) with energy levels in particular taking the longest to recover. Many of these patients will undergo adjuvant chemotherapy during this recovery period which can impact further on their quality of life. A proportion of patients never regain their baseline quality of life following treatment.

The length of the recovery period from surgery means that there is no role for oesophagectomy in patients with metastatic oesophageal cancer. There is no survival benefit by undergoing resection. The median survival for patients with metastatic or locally advanced oesophageal adenocarcinoma undergoing palliative chemotherapy is approximately nine months (Webb *et al.*, 1997) which is less than the time taken to recover baseline quality of life after surgery. All surgery is, for these reasons, performed with curative intent, which contrasts with the majority of other gastrointestinal cancers for which palliative resections are frequently performed.

Endoscopic mucosal resection

Patients who present with early, stage T1 oesophageal adenocarcinoma may be suitable for endoscopic mucosal resection. This endoscopic technique involves a submucosal injection of fluid that ‘lifts’ the mucosa and submucosa away from the muscle layers of the oesophagus. A piece of the mucosa and submucosa approximately one centimetre in diameter is then removed using cautery. This procedure can be considered a safe treatment for patients with stage T1a cancers that are confined to the mucosa and achieves good long-term oncological outcomes (Pech *et al.*, 2014). Oesophagectomy is not required for these patients because patients with T1a disease should not have nodal metastasis (Griffin *et al.*, 2011) and hence all of the disease can be removed endoscopically. Unfortunately, the number of patients who present with early oesophageal adenocarcinoma remains low, with only approximately ten per cent of patients staged with T1a disease. Patients with stage T1b tumours (involving the submucosa) may also be treated with this approach however in this group of patients the risk of lymph node metastases is approximately 12% (Griffin *et al.*, 2011). Endoscopic resection alone risks leaving disease behind

and therefore it is usual practice to offer all such patients with T1b disease a subsequent oesophagectomy.

1.1.8 Non-surgical Treatments

An important improvement in the outcomes of patients undergoing oesophagectomy for adenocarcinoma has been the development of peri-operative chemotherapy and chemoradiotherapy. The UK based MAGIC trial demonstrated a 13% five year overall survival benefit for patients with locally-advanced adenocarcinoma of the lower third of the oesophagus, junction or stomach who were treated with three cycles of neo-adjuvant ECF (epirubicin, cisplatin and fluorouracil) chemotherapy and three cycles of adjuvant ECF chemotherapy compared with those who underwent surgery alone (Cunningham *et al.*, 2006). The results of this study have formed the basis of current treatment guidelines in the UK. Other countries in the West have favoured the use of pre-operative combination chemo-radiotherapy. The Dutch CROSS trial demonstrated a significant overall survival benefit for patients treated with neo-adjuvant carboplatin and paclitaxel chemotherapy in combination with radiotherapy (41.4 Gy in 23 fractions) compared with patients undergoing surgery alone (van Hagen *et al.*, 2012). A new trial (Neo-AEGIS) comparing the CROSS and MAGIC protocols is currently recruiting within the United Kingdom (Keegan *et al.*, 2014).

A small proportion of patients with oesophageal adenocarcinoma may be offered curative treatment with either radical radiotherapy or radical chemo-radiotherapy. It is accepted that the outcomes from such an approach are inferior to those of surgery, with or without peri-operative chemotherapy. Radical radiotherapy or chemo-radiotherapy is therefore used as treatment in patients who are not fit for oesophagectomy or who do not wish to undergo surgery. It is only an option in patients with a relatively locally confined tumour. An early study of radical radiotherapy included patients with oesophageal adenocarcinoma and squamous cell carcinoma and reported three and five year survival rates of 27% and 21% (Sykes *et al.*, 1998). The addition of chemotherapy to radical radiotherapy leads to a further improvement in survival with five year survival rates of 30% reported (AlSarraf *et al.*, 1997).

Patients who have advanced, incurable disease at the time of diagnosis may be treated with palliative chemotherapy or palliative radiotherapy. These same treatments may be appropriate for patients who are not fit enough for major surgical resection. A proportion of patients are not sufficiently fit for any active treatment.

1.1.9 Outcomes

Historically, outcomes following oesophagectomy for adenocarcinoma were dismal. Post-operative mortality rates exceeded 20 per cent and disease recurrence rates were high. The one year survival following oesophagectomy in 1990 was as low as 21**%** (Muller *et al.*, 1990). Outcomes have fortunately improved steadily over the last twenty years. Improvements likely result from the centralisation of services, with fewer centres treating a greater number of patients, more accurate disease staging, better peri-operative care and improved neo-adjuvant and adjuvant oncological treatment. The UK national post-operative 90 day mortality rate is now only 3.2%. High volume centres now achieve five year survival rates of between 40-50%. Survival is related to the stage of disease, with early stage cancers having much more favourable outcomes than more advanced stages**.**

1.1.10 The Northern Oesophago-Gastric Cancer Unit

The Northern Oesophago-Gastric Cancer Unit (NOGCU) is the largest centre for the management of both oesophageal and gastric cancer in the United Kingdom (Figure 1.8). Approximately 300 patients with oesophageal cancer are staged each year and 100 oesophagectomies performed. On average two thirds of patients with oesophageal cancer have oesophageal adenocarcinoma.

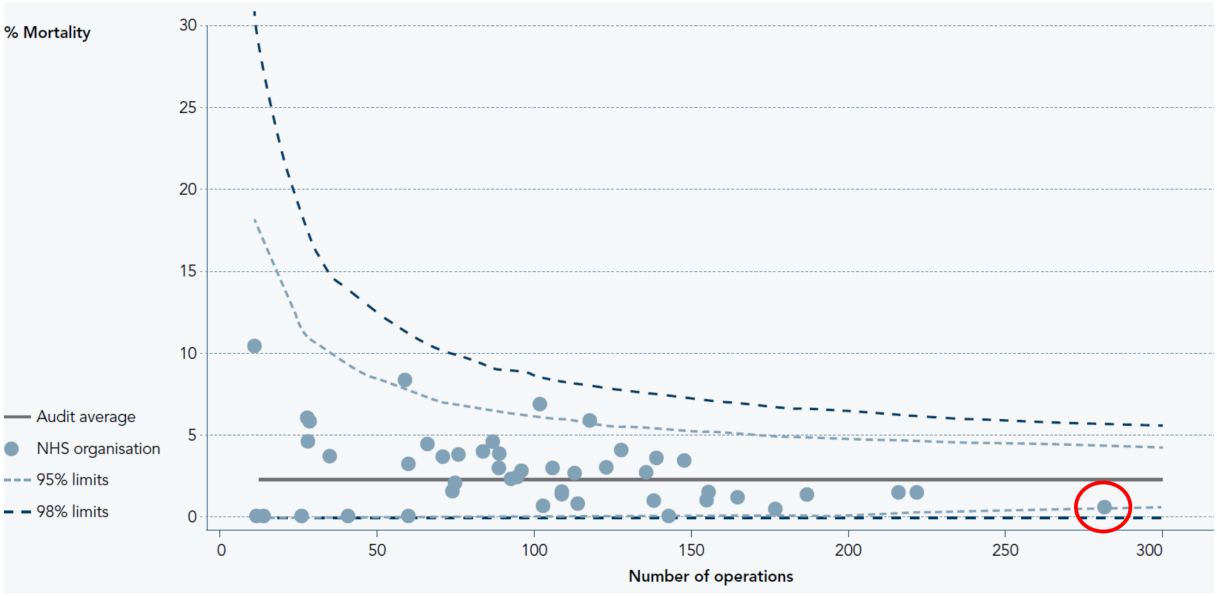


Figure 1.8. Funnel plot of the number of resections performed and 30 day mortality rates for all UK oesophago-gastric surgical units.

The 30 day mortality rates against the number of patients treated with surgical resection are illustrated. The mortality rate across all units combined (audit average) is demonstrated by the straight dotted line. The Northern Oesophago-Gastric Cancer Unit (NOGCU) which performs more resections than any other unit is identified by the red circle has a mortality rate below the national average. Modified from (*National Oesophago-gastric Cancer Audit - 2015*)

Within the NOGCU, outcomes following oesophagectomy have improved steadily. In the last 20 years, a total of 1047 patients have undergone oesophagectomy. Amongst these patients the overall five year survival is 38% (Dent *et al.*, 2014). Analysis of the whole period in five year cohorts demonstrates that overall survival has improved steadily with time. For those patients included in the most recent five year cohort, the overall five year survival following oesophagectomy is 49%. In-hospital mortality following oesophagectomy over the same time 20 year period has fallen from 7.5% to 1.9%.

1.1.11 Disease recurrence

Patients who undergo oesophagectomy for adenocarcinoma are at risk of disease recurrence either locally at the site of resection or in the form of distant metastatic disease. This metastatic disease is due to spread of tumour cells via the circulation.

Approximately half of patients have distant disease at the time of diagnosis of their recurrence (Dresner *et al*., 2000). Unfortunately despite rigorous radiological staging, a proportion of patients develop recurrent disease early during the post-operative recovery. Given the lengthy recovery period following oesophagectomy, recurrence in the first six to 12 months after surgery may be viewed as a failure of staging and treatment. Patients who recur within this timescale may be considered for palliative chemotherapy but only if they have made a sufficient recovery from surgery to be able to withstand this additional treatment. It is likely that patients who develop early recurrence following surgery would have had an improved quality of life if they had received palliative chemotherapy alone.

The higher the stage of disease the higher the risk of disease recurrence. Attempts have been made to identify risk factors within stage groups for very early recurrence. In oesophageal squamous cell carcinoma, tumour size and number of involved lymph nodes have been reported to be associated with early recurrence (Matsumoto *et al*., 2013: Shimizu *et al.*, 2012). At present these associations are not sufficient strong to be used to alter management decisions on an individual case basis.

1.2 Circulating Tumour Cells

Circulating tumour cells are created from the intravasation of cancer cells into the blood stream from primary or metastatic tumours (Figure 1.9).

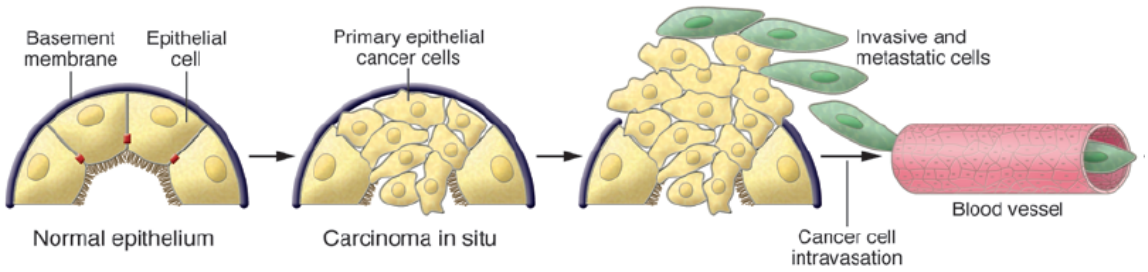


Figure 1.9. Schematic representation of the development of circulating tumour cells.

Following cancer cell invasion, intravasation occurs leading to the release of free tumour cells into the bloodstream. Adapted from (Kalluri and Weinberg, 2009)

Haematogenous spread is an important means by which tumours disseminate and develop distant metastatic sites. Metastatic disease plays a major role in determining

cancer prognosis. The study of circulating tumour cells aims to better understand and identify the development of this metastatic disease.

The ability to detect individual tumour cells within the bloodstream was first reported by Thomas Ashworth in 1869 in Australia (Ashworth, 1869). Individual tumour cells were discovered post-mortem in the femoral vessels of patients who had died of cancer. These cells were morphologically identical to those from the primary tumour.

One of the appeals of circulating tumour cell analysis as a means of studying and understanding tumour dissemination is the ease of obtaining samples from patients. Simple peripheral blood venepuncture is a routine part of the treatment of all cancer patients and additional sampling required for circulating tumour cell analysis poses no additional risks to patients. By contrast, biopsy of metastatic tumour sites frequently involves painful and technically demanding procedures that are invasive and carry significant risks to the patient. A major challenge in circulating tumour cell analysis however is the large number of normal blood cells amongst which a very minor population of tumour cells must be identified. There may be as few as one circulating tumour cell per one billion normal blood cells (Table 1.3). The white blood cell population is particularly relevant as these cells are the closest in size and density to the circulating tumour cell population. The ability to investigate circulating tumour cells as both a research and clinical tool depends on the development of reliable technologies with which to identify circulating tumour cells. It is vital that any method used to detect circulating tumour cells is sensitive enough to identify this rare cell population and specific enough to provide confidence in the cells being detected.

Type of cell	Number of cells per ml of fresh blood
Erythrocyte (Red blood cell)	5,000,000,000 (range from 4.4 to 5.9 x 10^9)
Leukocyte (White blood cell)	7,000,000 (range from 3.9 to 10.6 x 10^6)
Platelet	295,000,000 (range from 150 to 440 x 10^6)
Total	5,302,000,000
Reported CTCs	Range from 0.2 to >1000

Table 1.3. The types and numbers of different types of cells in human blood

1.2.1 Methods of circulating tumour cell detection

In response to the growing interest in circulating tumour cells over the last decade, a large number of techniques have been developed to identify circulating tumour cells. The ideal method for analysis of circulating tumour cells would be one in which all cells in the blood could be imaged and analysed. To achieve such an approach is unrealistic in view of the number of cells involved and hence enrichment of the sample is required. Enrichment aims to reduce the number of normal blood cells analysed without affecting the circulating tumour cells.

Enrichment relies upon differences in characteristics of the circulating tumour cells and normal blood cells. Broadly speaking, enrichment may consist of positively selecting the circulating tumour cells based on specific characteristics, or positively depleting the normal blood cells. Selection may be based upon physical, for example size, density or charge of the cells, or biological characteristics, for example specific antigen expression. In an ideal situation, enrichment would separate the circulating tumour cells from all other cells in the blood without losing any of the circulating tumour cells from the sample. In reality methods of enrichment are a balance between the purity of sample achieved and the loss of circulating tumour cells. The percentage of circulating tumour cells that remain after enrichment represents the recovery during the process. Following enrichment, the circulating tumour cells are enumerated based upon characterisation of the cells that distinguishes them from

any residual blood cells. This characterisation is most commonly achieved by analysis of antigen expression by immunofluorescence or immunocytochemistry.

A review published in 2012 discussed 43 technologies that had been developed or were in the process of being developed to enrich, enumerate or characterise circulating tumour cells (Parkinson *et al.*, 2012). Some methods of enriching samples are not followed by characterisation of the circulating tumour cells (Vona et al. 2000; Desitter et al. 2011). The authors of the review acknowledged that the assays have not been compared directly. With so many available methods, blood from a single patient may give different results depending upon which approach is used. This variability is a major disadvantage when trying to encourage clinicians to adopt the evaluation of circulating tumour cells in their clinical practice as it will undoubtedly undermine confidence. More information is required to understand which technologies are best, or perhaps more appropriately, which technologies are best for differing aspects of circulating tumour cell assessment.

The majority of clinical studies reported have relied on positive selection of tumour cells that express a single biomarker (Tibbe *et al.*, 1999; Nagrath *et al.*, 2007; Maheswaran *et al.*, 2008; Sequist *et al.*, 2009; Talasaz *et al.*, 2009; Gleghorn *et al.*, 2010; Stott *et al.*, 2010). The antigen chosen most commonly is epithelial cell adhesion molecule (EpCAM).
The only clinically approved method for circulating tumour cell enrichment and detection is the CellSearch® platform. This platform received FDA approval in 2008 for use in predicting prognosis in patients with metastatic breast, colorectal and prostate cancer.

1.2.2 CellSearch® (Veridex)

The CellSearch® platform relies upon positive selection of circulating tumour cells using magnetic beads conjugated to antibodies against EpCAM. The method provides both enrichment and limited characterisation of circulating tumour cells. The objects selected are detected with fluorescently-conjugated antibodies against cytokeratins 8, 18+ and/or 19+ and CD45 and with a nuclear stain, DAPI. Cells are loaded into a magnetic cartridge that pulls the cells to a single focal depth where they are imaged. Circulating tumour cells are identified through visual inspection of the images generated and are defined as objects that express cytokeratin and have a

nuclear signal but do not express CD45. Developed in the late 1990s, CellSearch® received FDA approval in 2008 for use in clinical practice. The quality of the circulating tumour cell images collected by the CellSearch® platform is illustrated in Figure 1.10.

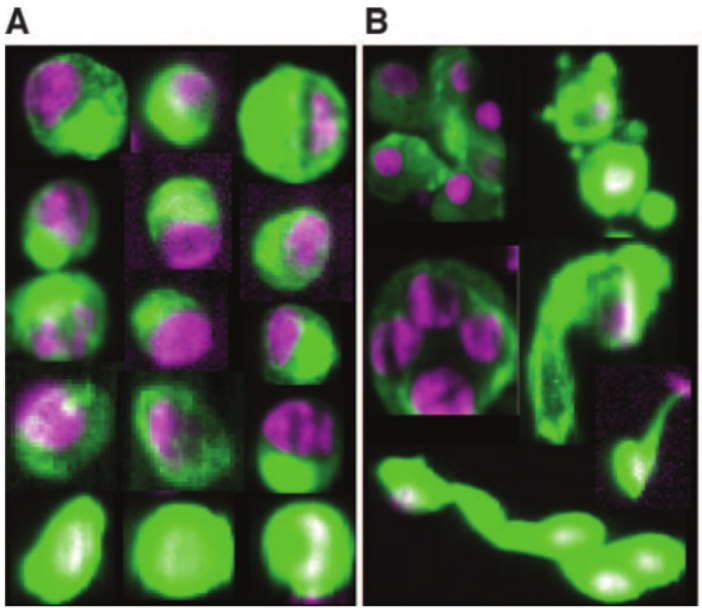


Figure 1.10. Examples of circulating tumour cell images captured using the Cellsearch® platform from blood samples of cancer patients.

Cells have been labeled with a fluorescently conjugated antibody to cytokeratin (green) and with DAPI (purple) nuclear stain. A. Examples of typical intact circulating tumour cells. B. Examples of clusters of circulating tumour cells or unusual shaped cells. Images reproduced from (Allard *et al.*, 2004)

The principle role of CellSearch® is the enumeration of circulating tumour cells. The concentration of circulating tumour cells detected with CellSearch® in patients undergoing treatment for metastatic breast, colorectal and prostate cancer has been shown to be associated with poor survival (Cristofanilli *et al.*, 2005; Cohen *et al.*, 2008; de Bono *et al.*, 2008). In breast cancer for example, a circulating tumour cell level of more than 5 cells per 7.5 ml of blood is associated with a poorer prognosis in terms of both disease free progression and overall survival than a circulating tumour cell level of 5 or less per 7.5 ml. (Cristofanilli *et al.*, 2004).

There are important considerations when interpreting the data obtained with the CellSearch® platform. Only objects that are EpCAM positive are captured by the CellSearch® platform. There is considerable heterogeneity in EpCAM expression in established epithelial cancer cell lines (Sieuwerts *et al.*, 2009), epithelial tumour cells may undergo epithelial mesenchymal transition (see section 1.3.2) and EpCAM expression may be influenced by cell cycle stage (Trzpis *et al.*, 2007). The EpCAM status of disseminated tumour cells in the bone marrow has been reported to change following chemotherapy (Thurm *et al.*, 2003).
Using the CellSearch® platform, circulating tumour cells have been detected in patients with metastatic disease of several different tumour types (Allard *et al.*, 2004). The number of circulating tumour cells detected varied considerably both between tumour types and between individual patients with the same tumour type.

By definition, circulating tumour cells should not be present in healthy people and patients with non-malignant diseases. Blood samples taken from 145 healthy women identified a circulating tumour cell in eight samples using the CellSearch® platform (Allard *et al.*, 2004). In the same study 14 out of 199 women with non-malignant breast disease also had at least a single detectable circulating tumour cell. The only explanation for such a finding is either non-malignant epithelial cells in the blood an inaccuracy in the classification of objects identified with CellSearch®. It is very difficult to be certain that atypical and clustered cells such as those demonstrated in Figure 1.10B are all in fact circulating tumour cells.

1.2.3 Imagestream^x (Amnis)

The Imagestream^x is an image flow cytometer that combines the capabilities of a fluorescence-activated cell sorting (FACS) machine with the additional benefits of high resolution microscopy. A total of 12 images of each cell are captured including brightfield, darkfield and up to ten fluorescent images at a rate of up to 5,000 cells per second. Cells can be labelled simultaneously with multiple fluorescently-conjugated antibodies against specific antigens. Combining the speed and sensitivity of FACS with the ability to characterise cells and identify subcellular locations of antigens makes the Imagestream^x image flow cytometer an extremely powerful tool for rare cell detection.

Cells in suspension pass into a flow cell and are focussed into a single stream. Illumination is provided by five lasers with excitation at 405nm, 488nm, 561nm, 642nm and 785nm. An infra-red laser provides an additional darkfield image. The fluorophores with which the antibodies are labelled are selected based upon their excitation by the different lasers and emissions. There are three objective lenses (x20, x40 and x60 magnification) through which light may pass. Light is then passed through two arrays of dichroic mirrors. Each set reflects light of different bandwidths and produces six separate images, giving a total of 12 images per cell which are captured by a two 12-bit charge coupled device (CCD) cameras.

The data captured by the Imagestream^{x} image flow cytometer is analysed with the IDEAS® software. This software allows selection of the imaged cells based upon various cell characteristics such as cell or nuclear area or specific antigen expression. This post-imaging selection facilitates the accurate identification of rare cell populations.

A single study has purported to compare directly analysis of the Imagestream^{x} and CellSearch platforms, after addition of PANC-1, pancreatic adenocarcinoma cells, to blood (Lopez-Riquelme *et al.*, 2013). The authors reported that at low PANC-1 cell concentrations, the accuracy of enumeration was lower with the Imagestream^{x} than CellSearch®. This study was however invalid because the authors used different methods of sample preparation and enrichment for the two platforms and analysed very small sample numbers. Different methods of sample preparation and enrichment may be associated with different losses of cells and hence differences in the total number of cells available to be detected. It is therefore very difficult to interpret the results from this study.

1.2.4 Reverse transcriptase-PCR

Not all circulating tumour cell research aims to identify individual cells. A common surrogate analysis is the detection of circulating tumour specific mRNA within the blood of patients by reverse transcriptase-PCR. The mRNA quantified is taken as a measure of circulating tumour cell levels. In the literature many authors report the detection of circulating tumour cells when they are actually reporting detection of circulating mRNA (Setoyama *et al.*, 2007; Cao *et al.*, 2009).

Using this approach, levels of mRNA detected have been reported to predict prognosis in patients with several different tumour types. Whole blood samples may be used although enrichment by density centrifugation is used frequently prior to the cell lysis step.

There are a number of concerns with the use of RT-PCR to analyse circulating tumour cells. RT-PCR has been demonstrated to have a high false positive rate of up to 29% in individuals who do not have cancer. A possible explanation for this is illegitimate gene expression in normal cells (Ko *et al.*, 2000) in which large numbers of normal cells expressing tumour specific markers at low levels.

1.3 Biomarker Selection

There are no reliable oesophageal adenocarcinoma specific biomarkers, which means that detection of circulating tumour cells in patients must rely upon biomarkers that would identify cells in patients with other types of cancer. Using multiple biomarkers to detect the cells is preferable to reliance on single antigen expression which does not take into account the potential for heterogeneity within the circulating tumour cell population. Analysis of multiple biomarkers increases the probability that circulating tumour cells will be detected or increases the percentage detected.

1.3.1 EpCAM

Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein that mediates calcium-independent cell-cell adhesion. It was first identified in 1994 (Litvinov *et al.*, 1994). It is expressed predominantly on the basolateral cell membrane of most human simple, pseudo-stratified and transitional epithelia. It is not expressed in squamous stratified epithelia or by non-epithelial cells.

Within the gastrointestinal tract there are marked differences in EpCAM expression (Balzar *et al.*, 1999). The normal oesophageal stratified squamous mucosa does not express EpCAM. EpCAM is detected in columnar epithelium at the gastro-oesophageal junction and within the glandular gastric mucosa. This differential expression of EpCAM is apparent in Barrett's metaplasia of the oesophagus in which

the normal squamous mucosa of the oesophagus is replaced by a glandular mucosa that expresses EpCAM (Wong *et al.*, 2006).

EpCAM is expressed in most carcinomas of epithelial origin but is not expressed by normal non-epithelial cells or by cancers of non-epithelial cells. EpCAM is distributed much more uniformly throughout the cell membrane in cancer cells than in normal epithelia because of the loss of polarisation of malignant cells. EpCAM is reported to play a role in tumour proliferation through the induction of gene transcription (Maetzel *et al.*, 2009). The role of EpCAM in tumour development is controversial. Conflicting evidence reports that increased expression of EpCAM may be linked to both improved and decreased survival across a range of tumour types (van der Gun *et al.*, 2010).

EpCAM expression has been demonstrated in oesophageal adenocarcinoma by immunohistochemistry (Kumble *et al.*, 1996; Went *et al.*, 2004; Anders *et al.*, 2008). In a study of 138 patients undergoing oesophagectomy, EpCAM expression was detected in 97.8% of the specimens (Kimura *et al.*, 2007). The authors reported a potential prognostic role for the level of EpCAM expression. Survival rates of patients with higher levels of EpCAM expression within the primary tumour were significantly higher than those with low-level expression. Further, high serum EpCAM mRNA levels measured by RT-PCR were an independent marker of good prognosis.

EpCAM is an ideal biomarker for circulating tumour cell detection as it is not expressed by any normal blood constituents. As discussed above, it is used for the initial selection of cells in the CellSearch® methodology.

1.3.2 Epithelial mesenchymal transition

Epithelial mesenchymal transition is the process by which cells of epithelial origin lose some of their normal epithelial phenotype and instead develop a mesenchymal phenotype. Epithelial cells lose their cell-cell adhesions and become motile and invasive. This process plays an important role in the development of invasive cancer and metastatic disease (Lee *et al.*, 2006). Metastatic cells within the bone marrow of patients with breast cancer have been demonstrated to show changes in protein expression in keeping with epithelial mesenchymal transition (Willipinski-Stapelfeldt *et al.*, 2005). There is not uniform agreement as to the role or importance of epithelial

mesenchymal transition within cancer development. The expected morphological changes are rarely seen in primary tumour specimens and histologically, metastatic and primary cancer tissue is very similar. These observations have led to speculation that epithelial mesenchymal transition may be a transient process which may be reversed following tumour cell dissemination (Brabletz *et al.*, 2001).

This change in phenotype is associated with changes in protein expression within individual epithelial cells. These changes in protein expression are important when considering the protein expression of circulating tumour cells. The role of EpCAM within the process of EMT is complex. There are marked differences in EpCAM expression between epithelial and mesenchymal tissues (Went *et al.*, 2004). EpCAM expression has been reported to be decreased in breast cancer cells that have undergone epithelial mesenchymal transition (Santisteban *et al.*, 2009). In oesophageal adenocarcinoma, a dynamic expression of EpCAM throughout tumour progression has been reported (Driemel *et al.*, 2014). High levels of EpCAM were associated with tumour proliferation where as low levels were linked to migration, invasion and dissemination.

The potential for the process of epithelial mesenchymal transition to affect studies of circulating tumour cells is significant, particularly for methods such as CellSearch® that depend entirely on EPCAM selection of circulating tumour cells. Such an approach may leave subpopulations of circulating tumour cells undetected (Gorges *et al.*, 2012). Epithelial mesenchymal transition has itself been reported in circulating tumour cells (Yu *et al.*, 2013) using methods of circulating tumour cell detection independent of EpCAM expression. In a study of 168 patients, with a variety of tumour types including gastric and colonic adenocarcinoma, circulating tumour cells were identified in 107 patients (Wu *et al.*, 2015). Epithelial, mesenchymal and mixed phenotype circulating tumour cells were identified in all tumour types. These findings highlight the importance of developing methods of circulating tumour cell detection that are able to detect heterogeneous subpopulations of cells.

1.3.2 Cytokeratins

Cytokeratins are proteins of keratin-containing intermediate filaments that are found in the cytoplasm of human epithelial cells. They form part of the intracytoplasmic

cytoskeleton. There are a large number of different cytokeratins and the relative expression of each is determined by the type of epithelium. All epithelia may be classified by their cytokeratin expression profile. When epithelia undergo malignant transformation, the cytokeratin profile remains unchanged. Cytokeratins associated with oesophageal adenocarcinoma include 7, 8, 18 and 19. (Moll *et al.*, 1982). A specific cytokeratin pattern (cytokeratin 7 positive and cytokeratin 20 negative) has been demonstrated as an effective method of distinguishing oesophageal from gastric adenocarcinomas (Driessen *et al.*, 2004).

Cytokeratins are not found in non-epithelial cells and hence are also not found in normal blood constituents. Like EpCAM, cytokeratins have been used widely for the detection of circulating tumour cells and their detection forms part of the identification of circulating tumour cells with the CellSearch® method. Measurement of circulating cytokeratin 19 mRNA has been used to estimate circulating tumour cell burden in oesophageal squamous cell carcinoma (Yin *et al.*, 2012).

1.3.3 Survivin

Survivin is an inhibitor of apoptosis (Fruscio *et al.*) and as such plays an important role in the regulation of cell apoptosis (Li *et al.*, 1998). It is also known as baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC 5). Survivin is not found in normal differentiated tissue but is expressed in the majority of tumours (Ambrosini *et al.*, 1997). Expression of survivin in tumours leads to the disruption of normal cell survival regulation. Survivin inhibits caspase 9 activation. If caspase 9 is not activated, its substrate pro-caspase 3 is not activated by cleavage, which in turn prevents PARP from being cleaved. PARP is cleaved during apoptosis both to prevent ongoing DNA repair but also to preserve NAD and ATP pools for the apoptotic process. Preventing PARP cleavage therefore prevents apoptosis from occurring. A common method by which chemotherapy drugs function is through the induction of apoptosis. The inhibitory effect of survivin on apoptosis is therefore a mechanism by which tumour cells may be drug resistant (Zaffaroni *et al.*, 2002).

Overexpression of survivin has been demonstrated in oesophageal adenocarcinoma cell lines and in human adenocarcinoma samples by both RT-PCR and immunohistochemistry (Malhotra *et al.*, 2013) (Rosato *et al.*, 2006). Inhibition of

survivin in cell lines resulted in an increase in apoptosis. Survivin expression has also been shown to increase in the progression through Barrett's metaplasia of the oesophagus to oesophageal adenocarcinoma. Immunohistochemical analysis of biopsies from 72 patients demonstrated an increase in both the intensity and extent of survivin expression as patients progressed from normal mucosa through non-dysplastic Barrett's mucosa, low grade dysplasia, high grade dysplasia and finally adenocarcinoma (Puccio *et al.*, 2015). The association of survivin expression with progression suggests a potential role for the inhibition of survivin to both prevent and treat oesophageal adenocarcinoma.

Circulating survivin mRNA detected by RT-PCR has been used to estimate circulating tumour cell burden in patients with both oesophageal squamous cell carcinoma (Cao *et al.*, 2009) and oesophageal adenocarcinoma undergoing surgical resection (Hoffmann *et al.*, 2007). In squamous cell carcinoma, high levels of survivin expression within the primary tumour are associated with an increased risk of tumour recurrence (Ikeguchi and Kaibara, 2002). High levels of expression in oesophageal adenocarcinoma have not been demonstrated to offer such prognostic information (Rosato *et al.*, 2006).

1.4 Clinical Value of Circulating tumour cells

The main focus of circulating tumour cell research has been how the concentration of circulating tumour cells relates to prognosis. Given the increasing clinical use of circulating tumour cells in patients with both metastatic and early stage breast cancer, it has been recommended that enumeration of circulating tumour cells should be included in the staging pathway for these patients. The 8th edition of staging classification from the American Joint Committee on Cancer (AJCC) for breast cancer includes circulating tumour cells within its definition of metastatic disease (Giuliano *et al.*, 2017). If circulating tumour cells are to be used in this way, they must provide additional prognostic information in addition to that already available from traditional radiological and histological staging. If the presence of circulating tumour cells is simply a marker of metastatic disease that has been detected by cross-sectional imaging for example, no additional information is provided by their inclusion in the staging pathway. Trials that have examined the value of circulating tumour cell enumeration in relation to prognosis frequently do not include a

comparison with radiological imaging (Cristofanilli *et al.*, 2005). Cross-sectional imaging of patients with metastatic cancer undergoing chemotherapy is a routine part of oncological follow up. Circulating tumour cell analysis must offer more than a surrogate marker of the radiological response to treatment.

The introduction of novel agents that target specific molecular characteristics of cancer cells has driven exploration for biomarkers with which to inform accurate stratification of patients. Current biomarker measurements rely upon surgical resection or invasive biopsies to obtain information about the biomarker profile of the primary tumour cells. Circulating tumour cells provide an opportunity to obtain a non-invasive biopsy from which information about the biological properties, or pharmacodynamic response to novel therapeutics, of disseminated malignant cells may be obtained.

1.4.1 CTCs in Oesophageal Cancer

There is less literature about the role of circulating tumour cells in oesophageal cancer compared with many other tumour types. Most studies have focused exclusively on oesophageal squamous cell carcinoma (Koike *et al.*, 2002; Tanaka *et al.*, 2010). This is largely because the studies originate from geographical regions where squamous cell carcinoma predominates. Whilst a recent meta-analysis has suggested poorer outcomes for circulating tumour cell-positive patients with squamous cell carcinoma (Wang *et al.*, 2017), results are reported frequently in mixed series of gastrointestinal cancers that commonly include gastric and colorectal cancers. (Huang *et al.*, 2003; Hoffmann *et al.*, 2007; Hiraiwa *et al.*, 2008) These mixed series make interpretation of the specific results for oesophageal cancer very difficult. Whilst oesophageal, gastric and colorectal cancers do all originate from the gastrointestinal tract they are very different diseases with very different outcomes and should not be analysed in combination.

The majority of studies have used RT-PCR to detect cell-free mRNA as a surrogate measure of circulating tumour cells. The most commonly used tumour markers are squamous cell carcinoma-associated antigen, carcinoembryonic antigen and survivin (Kaganoi *et al.*, 2004; Liu *et al.*, 2007; Cao *et al.*, 2009). Studies have also reported the detection of Np63 protein expression by RT-PCR, and detection of p16, E-

cadherin and RARβ expression by methylation-specific polymerase chain reaction (MSP) (Koike *et al.*, 2002; Ikoma *et al.*, 2007).

A number of studies have reported the detection of circulating cancer specific mRNA in patients with squamous cell carcinoma without obvious metastatic disease (Nakashima *et al.*, 2003; Kaganoi *et al.*, 2004; Setoyama *et al.*, 2007; Hashimoto *et al.*, 2008). The majority of these studies have sampled patients prior to oesophagectomy. One weakness of these studies is that they all include patients who have undergone differing neo-adjuvant chemotherapy or radiotherapy regimens. Direct comparison of results is therefore difficult and there is significant variation in the number of patients found to be positive for circulating mRNA prior to surgery. Detection rates of circulating mRNA of between 9.3% and 33% of oesophageal squamous cell carcinoma patients have been reported.

During or immediately following oesophagectomy, a higher proportion of patients are found to be positive for circulating mRNA (Nakashima *et al.*, 2003; Kaganoi *et al.*, 2004). This increase suggests that the process of handling the tumour during surgery may lead to shedding of tumour cells into the circulation. Such tumour cell dissemination mirrors results of similar studies of the impact of surgical resection in colorectal and gastric cancer (Ikeguchi and Kaibara, 2005; Park *et al.*, 2012). As discussed earlier, it is impossible to avoid some tumour handling during oesophagectomy.

Several studies have attempted to correlate circulating mRNA measurements with clinical outcome. All of these studies have relatively small numbers of patients and hence statistical adjustment for other confounding known influences on survival is difficult. Nevertheless, higher recurrence rates and reduced survival have been reported in patients with circulating mRNA compared to those without (Hashimoto *et al.*, 2008) (Cao *et al.*, 2009).

There are very few studies in oesophageal squamous cell carcinoma looking at the detection of circulating tumour cells themselves rather than circulating mRNA. Using the CellSearch® platform, circulating tumour cells have been detected in patients with both non-metastatic and metastatic oesophageal squamous cell carcinoma prior to commencing treatment (Hiraiwa *et al.*, 2008). Unfortunately, this study compared combined gastrointestinal tumours and so further interpretation is difficult. Another study using CellSearch® reported the value of palliative chemotherapy or

chemoradiotherapy in 38 patients with metastatic or recurrent oesophageal squamous cell carcinoma (Tanaka *et al.*, 2015). Circulating tumour cells at first follow up were independently predictive of survival.

The literature about circulating tumour cells in oesophageal adenocarcinoma is even more limited. A study in which survivin mRNA was measured by RT-PCR to detect circulating tumour cells in patients with a variety of gastrointestinal tumours included eight patients with oesophageal adenocarcinomas. Across all tumour types and locations, the levels of pre-operative survivin expression were higher than those measured after resection of the primary tumour in two thirds of the patients. Such a combined approach to the analysis makes interpretation of oesophageal adenocarcinoma specific results very difficult (Hoffmann *et al.*, 2010).

A study of circulating tumour cells in 43 patients with oesophageal adenocarcinoma or oesophageal squamous carcinoma without metastatic disease included 14 patients with resectable adenocarcinoma (Bobek *et al.*, 2014). Circulating tumour cells were detected in 11 of these 14 patients (78.6%). Circulating tumour cells were detected using a size-based filtration method and classified as tumour cells based on morphological analysis. Isolated circulating tumour cells were placed into culture and a maximum culture time of 14 days was achieved. There are a number of important concerns about this study. The patients studied include type 3 junctional tumours which are better considered as gastric cancers. Data on the staging process that patients underwent is not provided. It is surprising that a higher proportion of patients with resectable cancer had circulating tumour cells than patients with more advanced non-resectable tumours.

The combining of junctional and gastric adenocarcinoma makes the interpretation of results difficult in another study of 62 patients with advanced junctional or gastric adenocarcinoma undergoing chemotherapy. This study included 25 junctional adenocarcinomas (Kubisch *et al.*, 2015). Circulating tumour cells were analysed by immunomagnetic enrichment of cells that express mucin 1 and EpCAM followed by real-time PCR analysis of keratin 19, mucin 1, EpCAM and survivin. Patients who had detectable circulating tumour cells at baseline had a significantly shorter median progression-free survival. Only 14 of the 25 junctional adenocarcinomas were type one tumours (table 1.2). Care must therefore be taken when extrapolating these results to patients with oesophageal adenocarcinoma.

A pilot study was designed to assess the levels of circulating tumour cells in patients with advanced oesophagogastric adenocarcinoma undergoing palliative chemotherapy and to measure the impact of treatment on the circulating tumour cell levels. The CellSearch® platform was used. Circulating tumour cells could be identified using this method however the study was ended prematurely after inclusion of only nine oesophageal and two junctional tumours due to the loss of commercial funding (Sclafani *et al.*, 2014). A similar study again using the CellSearch® platform measured circulating tumour cell levels in a small group of patients with either oesophagogastric adenocarcinoma or pancreatic adenocarcinoma (Piegeler *et al.*, 2016). Whilst they found circulating tumour cells in seven out of eight pancreatic cancer patients, only one of the eight oesophagogastric patients had circulating tumour cells detected.

1.4.2 The potential role for circulating tumour cells in the management of oesophageal adenocarcinoma

For circulating tumour cells to become a clinically relevant tool they must add value to the management of patients. Where this value is added will differ between tumour types and the stage of patients' disease. There is great potential for circulating tumour cells to play an important role in the management of oesophageal adenocarcinoma. There are however a number of important considerations.

At present treatment options for patients with advanced oesophageal adenocarcinoma are limited. The median survival for patients with metastatic adenocarcinoma is nine months (Webb *et al.*, 1997). Palliative chemotherapy offers a modest median survival benefit of five to six months. There are few chemotherapy regimens available for this treatment. The use of circulating tumour cells purely as a means of predicting survival in such a cohort of patients may be limited. Circulating tumour cell enumeration may inform that a patient is in a group that on average will do better or worse. This enumeration is unlikely to provide accurate prognostic information for a single patient and should not be regarded as representing personalised medicine. It may be hard to justify the cost and time required for routine circulating tumour cell testing in this context.

It is possible however that in the future circulating tumour cell detection and characterisation may be used to predict response to palliative chemotherapy or to identify patients who are not responding to treatment. In this way patients who will, or are receiving, no benefit form chemotherapy agents can be identified and hence not be exposed to unnecessary side effects. This would have a major impact on quality of life in this patient group by allowing patients to switch to second line agents or to have their chemotherapy stopped altogether.

Circulating tumour cells may provide information about the biology of a tumour that could be used to help guide treatment decisions. There is growing interest in the potential use of anti-HER2 treatments in the management of oesophageal adenocarcinoma. The recent STO3 trial included a sub-study of the feasibility of including a trastuzumab treatment arm in the neo-adjuvant treatment of patients with resectable oesophageal and junctional tumours (Smyth *et al.*, 2016). Analysis of HER2 expression by circulating tumour cells may provide a non-invasive means to confirm the HER2 status of patients with advanced disease. Currently HER2 testing is performed on biopsies which are usually of the primary tumour. HER2 status in circulating tumour cells has been demonstrated to differ from that of the primary tumour in a large number of patients with breast cancer. (Fehm *et al.*, 2010). The number of patients with a HER2-negative primary tumour but HER 2-positive circulating tumour cells was between 32 % and 48 % depending on the circulating tumour cell assay used. Differences have also been reported in oesophageal cancer. Out of a total of 97 patients with oesophageal adenocarcinoma 14% had a HER2-positive primary tumour. The HER2 status of involved nodal disease and distant metastatic disease matched that of the primary tumour in 95 % and 86 % of cases respectively. (Schoppmann *et al.*, 2011). The authors concluded that routine sampling of metastatic tissue to obtain HER2 status is not warranted in all patients because taking biopsies from metastatic lesions is frequently technically difficult, painful and associated with significant risks to patients. It is possible that the HER2 status of circulating tumour cells will be a much closer match to that of metastatic tissue deposits than the status of the primary tumour. This is likely to be of particular relevance where there is a time lapse between treatment of the primary tumour and development of metastatic disease.

Another potential role for circulating tumour cells in oesophageal cancer is at a much earlier stage of the disease process. Circulating tumour cell detection may become a

part of the staging process for oesophageal adenocarcinoma as is recommended in breast cancer. Circulating tumour cell detection in patients who have no radiological evidence of metastatic dissemination may help to identify patients at risk of early disease progression. For patients undergoing oesophagectomy, this would include those patients at risk of early disease recurrence. Oesophagectomy has no role in the palliative setting but some patients who undergo surgery with curative intent, develop recurrence within 12 months of their operation. There is no question that if this recurrence could be predicted these patients would be better served being treated with palliative chemotherapy alone rather than undergoing surgery with the associated major impact that surgery has on quality of life for nine to 12 months post-operatively. Circulating tumour cell concentrations may also have the potential to guide the selection of patients for neo-adjuvant treatment and those who would be better undergoing unimodality treatment with surgery. They may provide a better marker of systemic response to neo-adjuvant chemotherapy than the current methods which rely heavily on either radiological or histological response.

1.5 Aims and objectives

1. Develop a novel method with which to detect, enumerate and characterise circulating tumour cells in patients with oesophageal adenocarcinoma.

2. Assess the concentration of circulating tumour cells in oesophageal adenocarcinoma patients without radiological evidence of metastatic disease.

3. Evaluate the potential of biological characterisation of circulating tumour cells in patients with oesophageal adenocarcinoma.

Chapter 2. Materials and methods

2.1 Commercial cancer cell lines

A panel of commercially available oesophageal adenocarcinoma cell lines were selected to validate the methodology for detection of circulating tumour cells. Several oesophageal cancer cell lines have been shown to be cell lines from other tumour types (Boonstra *et al.*, 2010). Only cell lines corresponding to true oesophageal or type one junctional tumours (see section 1.1.5) were selected. Details about the origin of the selected cell lines are included in table 1. All of the selected cell lines grew as adherent cultures with the exception of the ESO-51 cell line which grew in suspension.

Cell line name	Histology	Origin of cell line
SK-GT-4	Well differentiated adenocarcinoma arising from Barrett's mucosa	Established in 1989 from an 89 year old Caucasian male.
OE33 (JROECL33)	Poorly differentiated adenocarcinoma arising from Barrett's mucosa	Established in 1993 from a 73 year old female
FLO-1	Adenocarcinoma	Established in 1991 from the primary tumour of a 68 year old Caucasian male
OAC-P4C	Adenocarcinoma (Initially classified as a junctional tumour. Since reclassified as non-Barrett's oesophageal adenocarcinoma)	Established in 1996 from the primary tumour of a 55 year old Caucasian male
OE19 (JROECL33)	Moderately differentiated type 1 junctional adenocarcinoma	Established in 1993 from the primary tumour of a 72 year old Caucasian male
ESO-51	Adenocarcinoma arising in Barrett's metaplasia	Established in 2000 from primary tumour of 74 year old Caucasian male

Table 2.1. Histology and origin of selected oesophageal adenocarcinoma cell lines.

2.2 Tissue Culture

All cell culture was performed in a containment level II laminar flow microbiological safety cabinet. SK-GT-4 (DSMZ, Braunschweig, Germany), OE33 (DSMZ, Braunschweig, Germany), OAC-P4C (DSMZ, Braunschweig, Germany), OE19 (DSMZ, Braunschweig, Germany), and ESO-51 (DSMZ, Braunschweig, Germany) oesophageal cancer cells were grown in RPMI media supplemented with 10 % foetal calf serum (FCS). FLO-1 (DSMZ, Braunschweig, Germany) cells were grown in DMEM media supplemented with 10% foetal calf serum. All other reagents were purchased from Sigma-Aldrich, Poole, UK, unless stated otherwise.
Cells were maintained in exponential growth at 37°C in a humidified atmosphere, supplied with 5% CO_2 and discarded after the 30^{th} passage. Cells were confirmed to be mycoplasma free at regular intervals (MycoAlert mycoplasma detection kit: Lonza, USA). An example of SK-GT-4 adherent cells in culture is included in Figure 2.1.

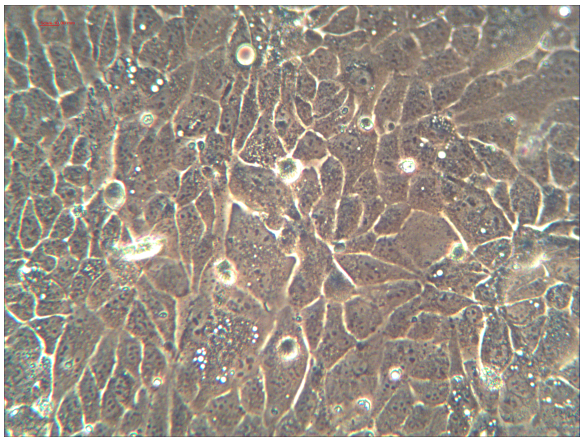


Figure 2.1. Oesophageal adenocarcinoma cell line in culture.

Example of confluent SK-GT-4 cells in adherent cell culture.

2.3 Immunofluorescence

SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and resuspended in fresh medium. Cells were counted with a haemocytometer and 1 x 10^6 cells were plated onto 400 mm^2 sterile coverslips in medium and incubated at 4^o C for 12 hours. The adherent cells were fixed with ice cold methanol for 20 minutes and then washed with 3 x 5 minute washes with phosphate buffered saline. Cells were blocked with 5% BSA in PBS solution and incubated in the dark with

conjugated antibodies against EpCAM (Alexa Fluor® 488 anti-human CD326 Clone: 9C4, Biolegend, Catalogue No. 324209.) and cytokeratins (Mouse anti-cytokeratin pan antibody, clone C11, FITC, Millipore, Catalogue No. CBL234F.) at a concentration of 1:100 for 1 hour at room temperature. Cells were washed for 3 x 5 minutes in phosphate buffered saline and mounted on glass slides with DAPI mounting media and imaged with a Leica DMR fluorescent microscope. Images were captured with Spot Advanced Software.

2.4 Image flow cytometry (Imagestream^{x})

Samples were analysed with an Imagestream^{X} (Amnis, USA) Mark II image flow cytometer (Figure 2.2) with an eight µm core at 60 mm/s with seven per cent Speed Beads®. Speed Beads® are a calibration reagent used to monitor the flow and focus of the Imagestream^{x} image flow cytometer The Imagestream^{x} image flow cytometer can process a volume between 20 and 200 µl. For cell line experiments, a volume of 60 µl was chosen. The Imagestream^{x} image flow cytometer has five lasers which excite at 405, 488, 561, 592 and 658 nm. Light emitted by the fluorescently-labelled cells is collected through an objective lens. There are three available objectives: x20, x40 and x60.

Two CCD cameras, each spatially resolved into 5 distinct spectral bandwidths, capture twelve images (six per camera) of each object passing through the core. These includes brightfield images and up to ten fluorescent images. Images are captured over a range of wavelengths between 430 and 745 nm. Channels 1 and 9 are reserved for brightfield images with the remaining ten channels capturing the fluorescent images. The maximal rate at which objects are imaged is dependent on the objective lens used. The rates are 4,000 objects per second at x20, 2,000 objects per second at x40 and 1,200 objects per second at x60. It is possible to capture all objects that pass through the core including the Speed Beads®. Alternatively objects meeting defined criteria, for example size or specific fluorescence, may be captured. Capture of objects with a diameter greater than 50 µm was selected to eliminate the Speed Beads® from the analysis whilst ensuring that no cellular objects were lost.

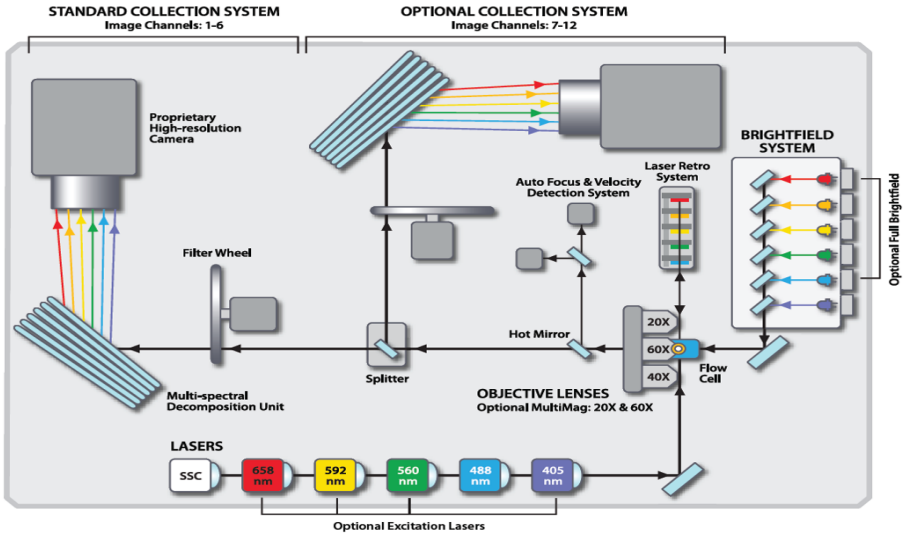


Figure 2.2 Imagestream^{x} Mark II image flow cytometer.

Schematic illustrating the mechanism of action of the Imagestream^{x} Mark II. The two CCD cameras capture twelve images (six per camera) of each object passing through the core contained within the flow cell. Five lasers excite the objects passing through the core at wavelengths from 405-642nm. (Images courtesy of STEMCELL Technologies™).

2.5 Compensation Matrix

There is spectral overlap between channels in the Imagestream^{x} image flow cytometer. An individual fluorophore will emit light that is predominantly captured within a single channel. There will also be some light captured in the adjacent channels. When using multiple fluorophores, this overlapping of light capture could lead to false positive results. A compensation matrix is therefore required for every sample processed on the image flow cytometer in order to remove this spectral overlap.

A compensation matrix is calculated after analysis of reference samples for each fluorophore being used. These reference samples are created by incubating cells with a single fluorophore. Only 500 cells are analysed for each reference single colour control sample. The fluorescence data for each reference sample is then combined by the IDEAS® software to create the final compensation matrix. When experimental samples are processed, this matrix must be applied to the raw image data before further sample analysis occurs.

2.6 Antibody labelling of samples for image flow cytometry

SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed by incubation in 4% formaldehyde in phosphate buffered saline for 20 minutes at room temperature, or Phosflow Lyse/Fix buffer (BD, Oxford, UK), and permeabilised by incubation in Perm/Wash buffer (BD, Oxford, UK) for one hour at room temperature. Cells were incubated with 1:20 anti-pan-cytokeratin (clone C-11) PE (Cayman Chemical, USA) and 1:50 anti-survivin Alexa Fluor® 647 (Cell Signalling, USA) in phosphate buffered saline for 30 minutes at room temperature. Membrane antibodies and nuclear stains were added and incubated for one hour at room temperature: 1:20 anti-CD45 (clone H130) V450 or PE:Cy7 (BD Biosciences, USA); 1:20 anti-EpCAM CD326 (clone 9C4) Alexa Fluor® 488 (Biolegend, USA); DAPI or DRAQ5 (Biostatus, UK) in phosphate buffered saline. Cells were washed in 500 µl of Perm/Wash buffer and recovered by centrifugation at 500 g for five minutes and either analysed immediately or stored as a pellet at 4 ^oC until analysis. The cells were resuspended in phosphate buffered saline in a minimum volume of 60 µl for analysis on the Imagestream^x image flow cytometer.

Cells were visualised with an Imagestream^X image flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x20, x40 and x60 objective with the wavelengths for the collection channels set at: 480-560 nm, EpCAM; 560-595 nm, cytokeratins; 642-745 nm, survivin; 745-800 nm, CD45; 430-505 nm, DAPI.

2.7 Analysis of patient samples

Whole blood samples were obtained from patients undergoing treatment for oesophageal adenocarcinoma at the Newcastle-upon-Tyne NHS Foundation Trust. Patients attended a staging clinic at the Northern Oesophago-Gastric Cancer Unit. As part of this clinic, all patients are reviewed by a surgeon and undergo routine blood sampling. Patients gave informed consent and had blood taken for analysis of sampled for circulating tumour cells at this clinic appointment. In this way patients were not inconvenienced by additional trips to hospital or extra blood tests. A single 8

ml sample of blood was taken from each patient, Routine blood samples were taken prior to the circulating tumour cell sample to act as the blood discard.

Objects were classified as circulating tumour cells if they were nucleated, with a cellular morphology and did not express CD45. To account for potential heterogeneity within the circulating tumour cell population all objects meeting these criteria were inspected irrespective of the expression of EpCAM, cytokeratin and survivin. Samples were inspected by a single person with no formal 'double reading'. Where doubt existed a second opinion was sought from with an individual with experience of circulating tumour cell research.

Ethical approval for the study was obtained from the Newcastle and North Tyneside Research Ethics Committee (Appendix 1). Blood samples were collected in Transfix (Cytomark, UK) collection tubes to store for up to 24 hours at 4 ^{o}C or BD Vacutainer EDTA tubes (BD Biosciences, USA) for immediate use. With all blood samples an initial draw of 4 ml of blood was discarded to prevent contamination with epithelial cells. All samples were processed within 48 hours of sampling to reduce the potential for any sample degradation. All patients were assigned an anonymised identification code. Patient clinical data was recorded for each patient in accordance with ethical approval. Follow up of patients was in accordance with routine clinical practice and consisted of regular clinical review without routine radiological or endoscopic examination. In addition whole blood samples were obtained from healthy volunteers (Appendix 2).

2.8 Recovery of cancer cells form whole blood

The SK-GT-4 cell line was chosen for the cell recovery experiments because this cell line easily trypsinises into predominantly single cells which ensures accurate cell counting. Confluent SK-GT-4 cells were trypsinised and fixed in 4% formaldehyde in phosphate buffered saline before being resuspended in phosphate buffered saline. Cells were counted with a Neubauer haemocytometer and diluted with phosphate buffered saline to a final concentration of 20,000 cells per ml. Initial experiments analysed a single concentration of 2000 cancer cells in five ml of whole blood collected from healthy volunteers. An aliquot of 100 µl of the prepared cell suspension was added to the blood sample. This concentration of 2000 cells per ml

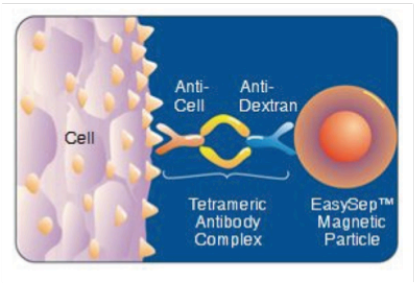
of blood is considerably higher than the expected concentrations of circulating tumour cells. Once the full protocol had been developed, further experiments analysed a range of cell concentrations. SK-GT-4 cells were trypsinised, fixed and resuspended. From a stock suspension of 20,000 cells per ml of blood two serial dilutions of one in 10 were made and 100 µl of each suspension were added to five ml of blood to give a total of 2000, 200 and 20 cells.

2.9 EasySep™

EasySep™ provides a column-free isolation method to purify cell populations based on specific cell membrane antigen expression. Cell populations can be positively selected based on their own membrane antigen expression or negatively selected by selecting other cell types from the sample. For the purpose of detecting circulating tumour cells, a negative selection was used. The white blood cells in the sample were depleted based on their expression of the membrane target antigen CD45.

Figure 2.3 demonstrates this depletion of white blood cells. Antibodies against CD45, bound in tetrametric complexes were added to the cell suspension in a 15 ml round bottomed Falcon tube and incubated for 15 minutes at room temperature (Figure 4A). Dextran-coated magnetic nanoparticles were added and incubated with the cell suspension for an additional ten minutes The cell suspension was placed within a specific EasySep™ 'Big Easy' magnet for ten minutes. The magnet and tube were inverted for a maximum of two seconds to pour off the un-retained cell suspension (Figure 4B). CD45 positive cells, representing the white blood cell population were retained within the tube by the magnetic field. The unretained cell suspension was centrifuged at 500 g for five minutes and the cell pellet resuspended in Perm/Wash buffer ready for analysis.

A.



B.

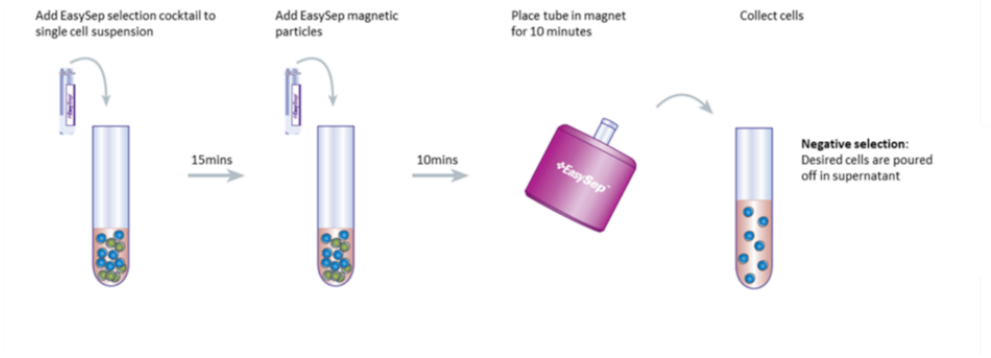


Figure 2.3. Depletion of white blood cells with the EasySep™

(A) The EasySep™ magnetic particles bind to the tetrametric antibody complex which is in bound to the surface target protein. (B) Following incubation, the magnet containing the round bottomed Falcon tube is inverted. (Images courtesy of STEMCELL Technologies™).

2.10 Final protocol for analysing patient samples

The finalised protocol for detecting circulating tumour cells in patient samples processing is described below. Details about the development of this protocol can be found in chapter 4.

Five ml of 5% bovine serum albumin in AutoMACS rinse solution (Miltentyi Biotec, Germany) was added to a 50 ml Falcon tube and rotated for 5 minutes. The bovine serum antigen solution was decanted from the Falcon tube and five ml of whole blood was added to the Falcon tube and stored at 4 ^{o}C for 15 minutes. 50 µl of human FcR blocking reagent (Miltenyi Biotec, Germany) was added to the blood and stored at 4 ^{o}C for a further 15 minutes. The FcR blocking reagent blocks the binding of antibodies to human FC receptor–expressing cells such as B cells, monocytes and macrophages, increasing the specificity of antibody binding to the cancer cells.

45 ml of BD Phosflow Lyse/Fix buffer 1:20 (v:v) (BD Biosciences, USA) was added to the blood sample and the Falcon tube inverted vigorously 8-10 times to mix the cell suspension and incubated at 37 ^{o}C for 10 minutes. The sample was centrifuged at 500 g for 8 minutes and the cell pellet resuspended in one ml of RoboSep™ buffer (STEMCELL Technologies, UK) and then transferred to a 14 ml round bottomed polystyrene Falcon tube with a siliconised pipette tip.

The original 50 ml Falcon tube was washed with one ml of RoboSep™ buffer which was then again transferred to the 14 ml Falcon tube with the same siliconised pipette tip. This washing of the Falcon tube was repeated a further three times. The 14 ml Falcon tube was then centrifuged at 250 g for 5 minutes and the pellet resuspended in 500 µl of RoboSep™ buffer.

25 µl EasySep™ whole blood CD45 depletion cocktail (STEMCELL Technologies, UK) was added to the cell sample with a fresh siliconised pipette tip and stored at 4 ^{o}C for 15 minutes. 50 µl of the EasySep™ magnetic nanoparticles (STEMCELL Technologies, UK) were added with a siliconised pipette tip and the sample stored at 4 ^{o}C for 10 minutes. 4.5 ml of RoboSep™ (STEMCELL Technologies, UK) buffer were added to the sample and the Falcon tube placed without the cap on in “The Big Easy” EasySep™ magnet (STEMCELL Technologies, UK) at room temperature for 10 minutes.

The supernatant was poured off for two seconds into a 15 ml Falcon tube and centrifuged at 500 g for 5 minutes. The cell pellet was resuspended in 200 µl of perm/wash buffer and transferred to a siliconised eppendorf using a siliconised pipette tip. The 15 ml Falcon tune was washed a further four times using 200 µl of perm/wash buffer and the same siliconised pipette tip. The sample was centrifuged at 500 g for 5 minutes and the pellet resuspended in 100 µl of perm/wash solution.

At this stage the sample was labelled for analysis on the Imagestreamx as described in section 2.6.

2.11 Statistical Analysis

The areas of individual cells were measured directly by the IDEAS® software. Data was presented as means ± standard errors of the mean. An unpaired t-test was used

to compare data between two groups and a one-way ANOVA for multiple groups. All graphs were produced using GraphPad Prism 6. Survival analysis was plotted as Kaplan-Meier curves using GraphPad Prism 6 and curves compared with log-rank testing.

Chapter 3. The use of image flow cytometry to detect and characterise oesophageal adenocarcinoma cells

3.1 Introduction

The ability to detect circulating tumour cells relies upon the accurate identification of the tumour cells. The circulating tumour cells must be easily distinguished from normal blood constituent cells. A definition of what constitutes a tumour cell, based upon morphological features or the expression of specific antigens, is required. The most widely used method in clinical practice is the CellSearch® platform which identifies circulating tumour cells based upon the expression of EpCAM and epithelial cytokeratins and the lack of expression of CD45 (Allard *et al.*, 2004). A number of different methods of identifying circulating tumour cells have however been described (Parkinson *et al.*, 2012).

The Imagestream^x image flow cytometer captures high resolution images of both cell morphology and cellular immunofluorescence and allows accurate characterisation of cells. It is able to capture these data whilst imaging up to 5000 cells per second. This chapter details the development of a method with which to identify and characterise oesophageal adenocarcinoma cells using the Imagestream^x image flow cytometer. Fluorescently-conjugated antibodies against specific antigens were identified and validated with a panel of oesophageal adenocarcinoma cell lines and white blood cells.

3.2 Results

3.2.1 Image acquisition and quality using Imagestream^x

Imagestream^x image flow cytometry combines high resolution immunofluorescence with the high-speed processing of flow cytometry. A comparison of the images produced with conventional immunofluorescence and with the Imagestream^x image flow cytometer and is shown in Figure 3.1. Images of EpCAM and cytokeratin expression by SK-GT-4 oesophageal adenocarcinoma cells detected by conventional immunofluorescence allow the different intra-cellular localisations of the membrane

bound EpCAM and cytoplasmic cytokeratins to be observed (Figure 3.1A and C). The cells are adherent to a glass coverslip and have a characteristic flattened polygonal morphology.

The image flow cytometer images individual cells in suspension. For each cell, images that incorporate fluorescence from the antibodies and nuclear dyes used are captured with two separate cameras in addition to brightfield images. Images of SK-GT-4 cells captured with an image flow cytometer are in suspension and demonstrate different morphology, with the cells appearing spherical (Figure 3.1 B and D). The images demonstrate the differences between the EpCAM and cytokeratin localisation. The EpCAM immunofluorescence is particularly high and is concentrated along the plasma membrane. The cytokeratin immunofluorescence is slightly less intense than the EpCAM immunofluorescence and is detected more evenly throughout the cytoplasm. The high quality images allow both the detection and localisation of target antigens. This is seen at all magnifications but is especially clearer when moving from x 20 to x 40 magnification. The difference between x 40 and x 60 magnification is as expected less pronounced (Figure 3.1 B). The images of individual cells are displayed in rows to allow easy visual comparison. The IDEAS® software allows for rapid qualitative and quantitative analysis and comparison of large numbers of these images. With traditional immunofluorescence, analysis of such large numbers of cells would be more difficult and labour intensive.

The greater the magnification, the higher the quality of images captured. The increased quality is at the expense of the speed of image acquisition. The maximum rate of imaging is 5000 cells per second at x 20 magnification, 2000 cells per second at x 40 and 1200 cells per second at x 60. For most applications x 40 magnification was chosen as a compromise between speed and image quality. The intracellular morphology can be appreciated easily at this magnification (Figure 3.1B. ii). Speed of processing is less important for experiments with cell lines but is a crucial consideration for analysis of patient samples (Chapter 4).

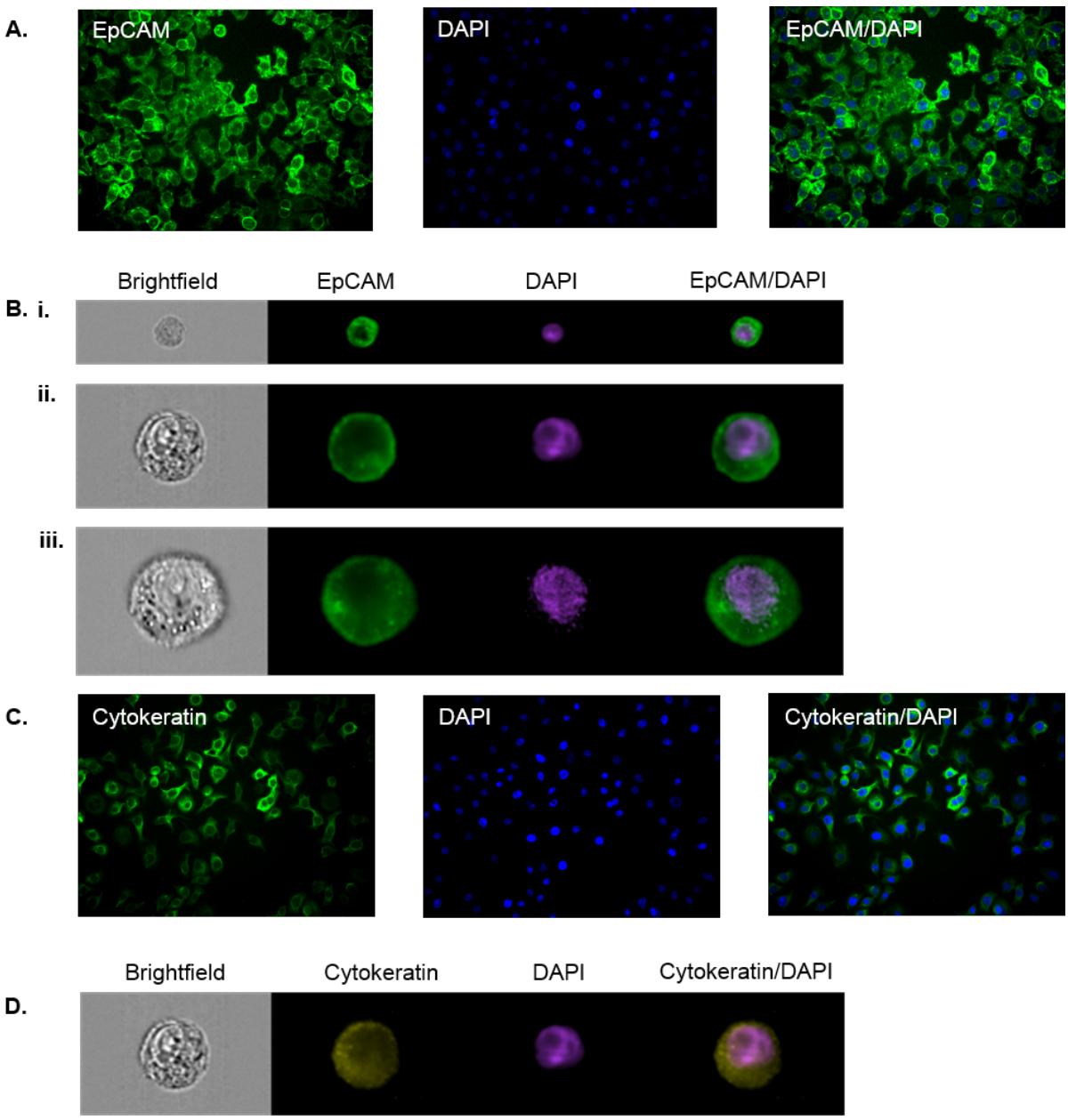


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Figure 3.1 Comparison of images captured by traditional immunofluorescence and Imagestream^{x} image flow cytometry.

A and C. SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and resuspended in fresh medium. Cells were counted using a haemocytometer and 1 x 10^6 cells were plated onto sterile coverslips in medium and allowed to adhere overnight. The adherent cells were washed and fixed with ice-cold methanol and washed with PBS. Cells were blocked with 5% BSA solution and incubated in the dark with conjugated antibodies against EpCAM (Alexa Fluor® 488) (A) and cytokeratin (FITC) (C) for 1 hour. Cells were washed and mounted on glass slides with DAPI mounting media and imaged using a Leica DMR fluorescent microscope. Images were captured with Spot Advanced Software. B and D. SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. Cells were permeabilised by incubation with 0.3 % saponin, incubated with a pan cytokeratin antibody and subsequently with DAPI (D) or incubated simultaneously with antibody against EpCAM and with DAPI (B). Cells were visualised with an Imagestream^{X} flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images of cells labelled with EpCAM and DAPI were collected with a x20 (B.i.), x40 (B.ii) and x60 (B.iii.) objective with the wavelengths for the collection channels set at: 480-560nm, EpCAM; 430-505nm, DAPI. Images of cells labelled with cytokeratin and DAPI were collected with a x40 objective with the wavelengths for the collection channels set at: 560-595 nm, cytokeratins; 430-505nm, DAPI (D).

3.2.2 Image Compensation

Cells labelled with a single fluorescently-conjugated antibody are detected primarily in a single one of the image flow cytometry collection channels. The extent to which the emission from each fluorophore is restricted to a single channel is dependent on the fluorophore used, the wavelength of the excitation laser and the wavelength of the collection channel. Figure 3.2 illustrates the channels in which common fluorophores are collected with the image flow cytometer.

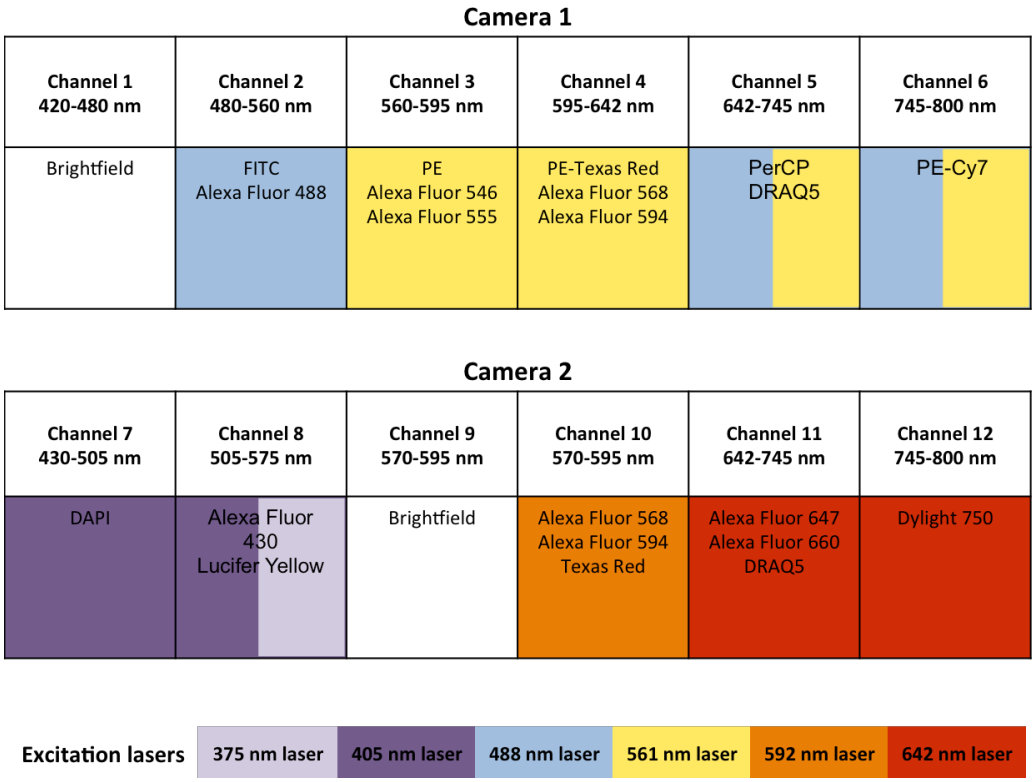

Figure 3.2. Common fluorophores and the corresponding excitation lasers and collection channels for the Imagestream^x image flow cytometer

Each camera captures six channels corresponding to overlapping wavelengths of emitted light. Examples of the common fluorophores that will emit light that will be captured in each channel are included in the figure. The wavelength of the laser excitation required for the emission for each fluorophore are also illustrated. Channels one and nine capture a brightfield image only. The maximum number of fluorphores that could be used simultaneously is therefore ten.

No single fluorophore has a sufficiently narrow emission spectrum to allow it to be collected (or for its emission to fall within) a single channel. Consequently, for any fluorophore there will be some collection of the fluorescence in other channels. This collection in other channels is called spectral overlap and is greatest in the immediately-adjacent collection channels and reduces as the collection channel wavelengths moves further away from that of the primary collection channel. Without correction, this spectral overlap would prevent the simultaneous analysis of multiple fluorophores. The adjustment of the data to analyse only signal collected within the primary detection channel is termed compensation.

Figure 3.3A illustrates the effect of compensation on the images captured by the image flow cytometer. The original images collected are from a cell labelled with a pan-cytokeratin antibody conjugated to PE (Figure 3.3Ai). Whilst the greatest fluorescence signal is seen in channel 3, fluorescence is also detected in channels 2, 4 and 5. There is no image in channel 1 as the brightfield image is not collected when acquiring the single colour controls. The data collected for the single colour control are analysed to calculate the average percentage of the total fluorescence that is collected in each channel. For each cell that is imaged this percentage of total fluorescence collected in the primary channel is subtracted from the fluorescence collected in the secondary channels.

Images of the same cell after compensation has been carried out illustrate that fluorescence is detected only in the primary channel (Figure 3.3Aii). A graphical representation of the effect of compensation is also provided.

The importance of compensation is clearer with analysis of multiple fluorophores. Figure 3.3B includes multiple images of a single cell without and with compensation having been applied. Without compensation fluorescent signal appears to be detected in all channels (Figure 3.3Bi) whilst after compensation is performed, signal is only visualised in those channels corresponding to the fluorophores being used (Figure 3.3Bii). When comparing the EpCAM and cytokeratin images individually, compensation leads to a slight reduction in the intensity of fluorescence but the expression is more specific and more easily localised within the cell. The membranous EpCAM for example is more easily identified after compensation.

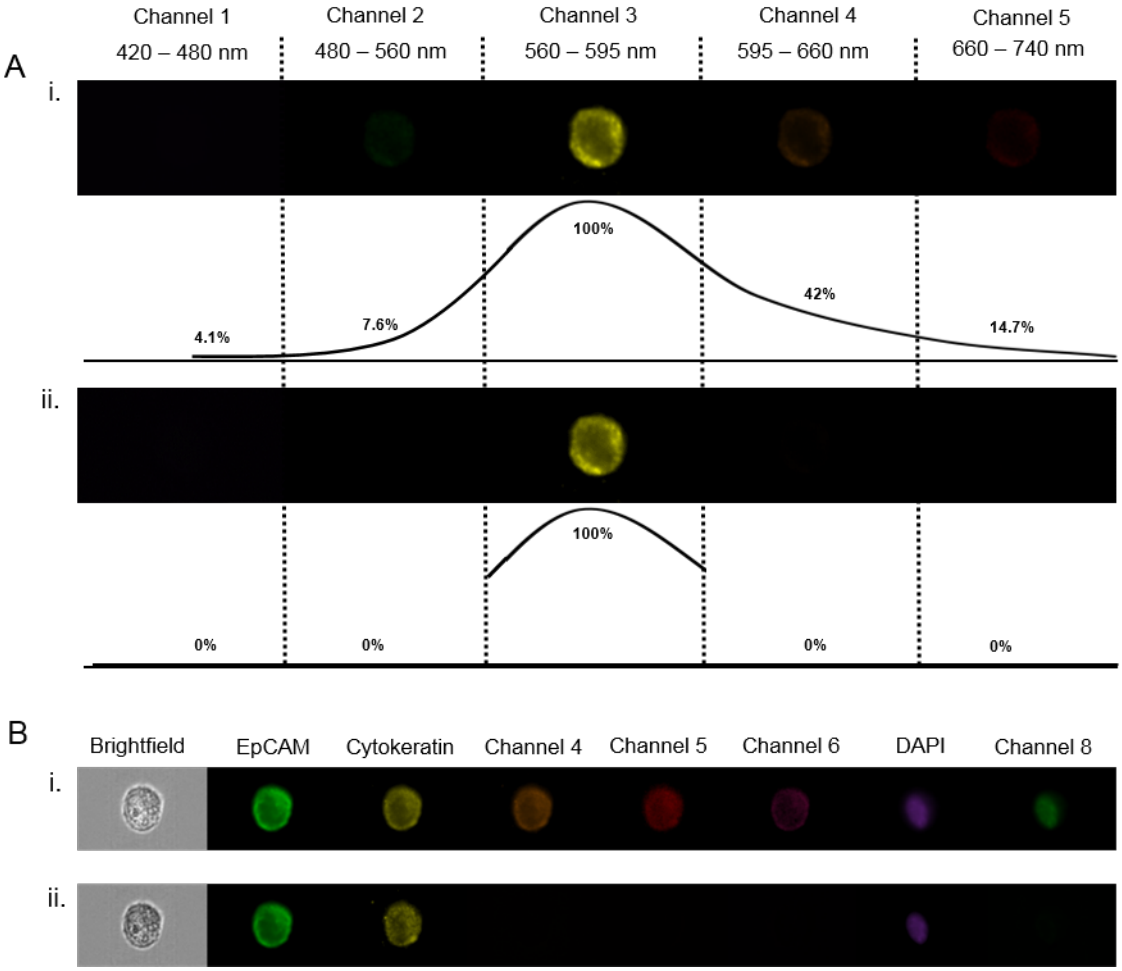

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Figure 3.3. The effect of compensation on image intensity using image flow cytometry

A. SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. Cells were permeabilised by incubation with 0.3 % saponin and incubated with antibody against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and incubated subsequently with DAPI. Single colour controls were generated for cytokeratins and for DAPI by incubation of further cells with each of these alone. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 560-595 nm, cytokeratins and 430-505nm, DAPI. Cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit at 405, 488, 561 and 658 nm. A compensation matrix was generated from the single colour controls using INSPIRE® software. An example of an imaged cell both before (i) and after (ii) the compensation matrix was applied is included along with a graphical representation of the percentage of the fluorophore detected in secondary channels as measured by the INSPIRE® software. B. SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. Cells were permeabilised by incubation with 0.3 % saponin and incubated with antibody against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and incubated subsequently with antibody against EpCAM and with DAPI. Single colour controls were generated for EpCAM, cytokeratins and for DAPI by incubation of further cells with each of these alone. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560nm, EpCAM; 560-595 nm, cytokeratins and 430-505nm, DAPI. Cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit at 405, 488, 561 and 658 nm. A compensation matrix was generated from the single colour controls using INSPIRE® software. An example of an imaged cell both before (i) and after (ii) the compensation matrix was applied is provided.

3.2.3 Image selection using image flow cytometry

Once images have been captured by the image flow cytometer it is important to identify appropriate cell populations for further analysis. Cell populations can be distinguished easily using the IDEAS® software (Figure 3.4.)

Consideration of the total area of each object and the aspect ratio of the object provides a simple means by which to identify the different cell populations as illustrated in Figure 3.4A. The aspect ratio describes the proportional relationship between the width and height of an object (width divided by height). Areas have been selected on the scatter plot to define four populations of cells (Figure 3.4B). The first area which encompasses the smallest objects corresponds to Amnis Speedbeads® (Figure 3.4Bi). As discussed in Chapter 2, these beads are part of the calibration of the image flow cytometer. They are not fluorescent. The second area contains objects that have an aspect ratio close to one and contain single cell images (Figure

3.4Bii) The highest density of objects is located within this selected population. The large numbers of cells within this population is in part a reflection of the SK-GT-4 cells which are easily trypsinised into a predominantly single cell suspension. This cell line is therefore ideally suited to analysis with the image flow cytometer.

The third and fourth areas represent pairs or groups of cells (Figure 3.4Biii and iv). These objects have a correspondingly larger area but lower aspect ratio. Whilst single cells were selected principally for analysis, the ability of the image flow cytometer to image groups of cells is important when considering imaging circulating tumour cells which may not all be single cells.

The populations illustrated in Figure 3.4 were gated using IDEAS® after image collection. It is possible to gate the images at the time of collection. In this way, only objects that lie within a defined area of the scatter plot are collected. This allows a very specific population of objects to be collected. The major disadvantage of this approach is that it may limit the heterogeneity of the sample and populations must be defined before the start of the collection process.

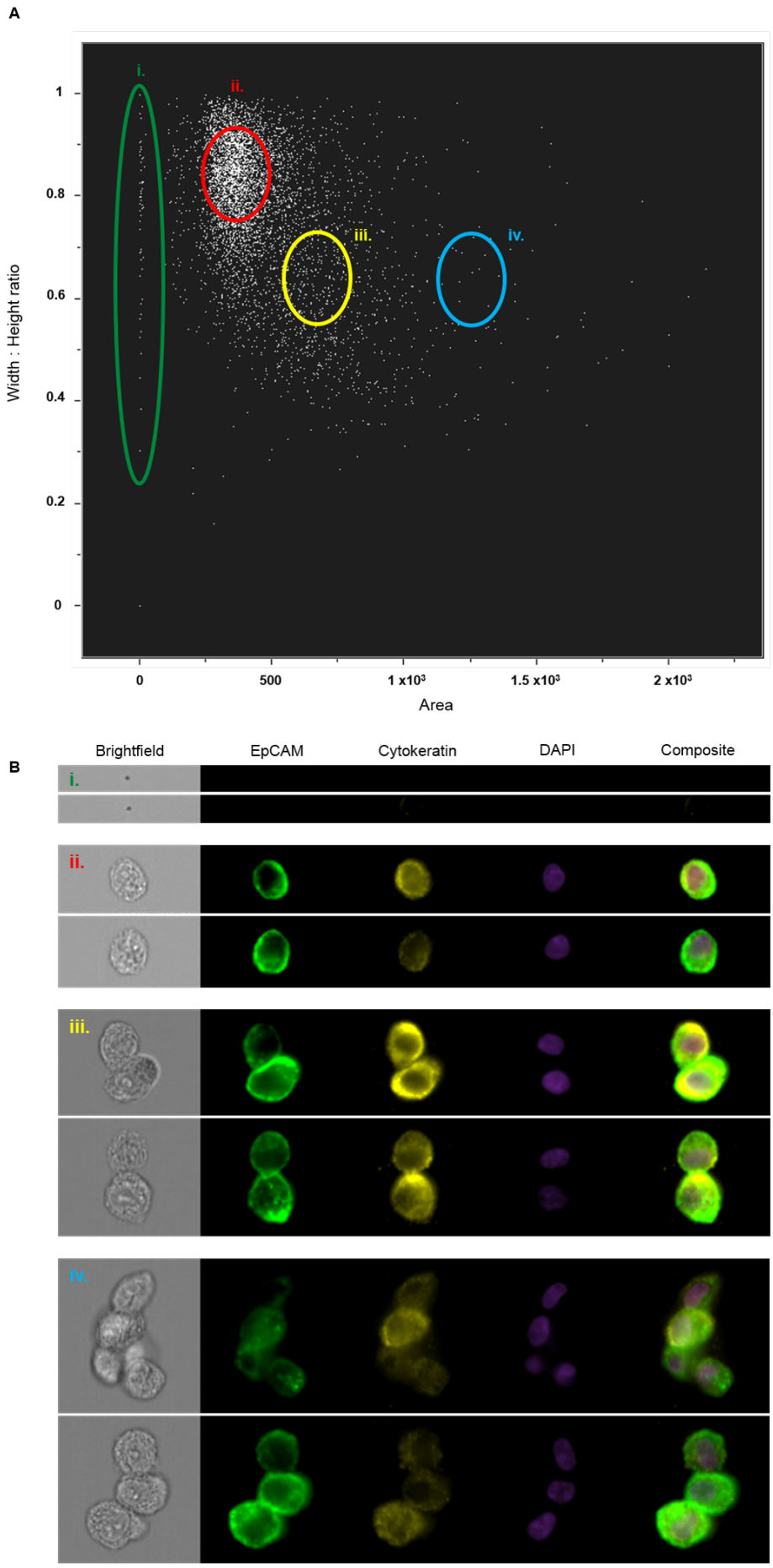

A
i.
ii.
iii.
iv.
Width : Height ratio
1
0.8
0.6
0.4
0.2
0
0
500
1 x10³
1.5 x10³
2 x10³
Area
B
Brightfield
EpCAM
Cytokeratin
DAPI
Composite
i.
ii.
iii.
iv.

Figure 3.4. Selection of single cell images using image flow cytometry

SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibody against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and incubated subsequently with antibody against EpCAM and with DAPI. Cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560nm, EpCAM; 560-595 nm, cytokeratins; 430-505nm, DAPI. A. Following image collection a scatter plot of object area against aspect ratio was generated using IDEAS® from which populations of objects were selected. B. Images of representative objects from each selected population are displayed. The coloured labels (i.- i.v) correspond to the selected populations of objects in figure A.

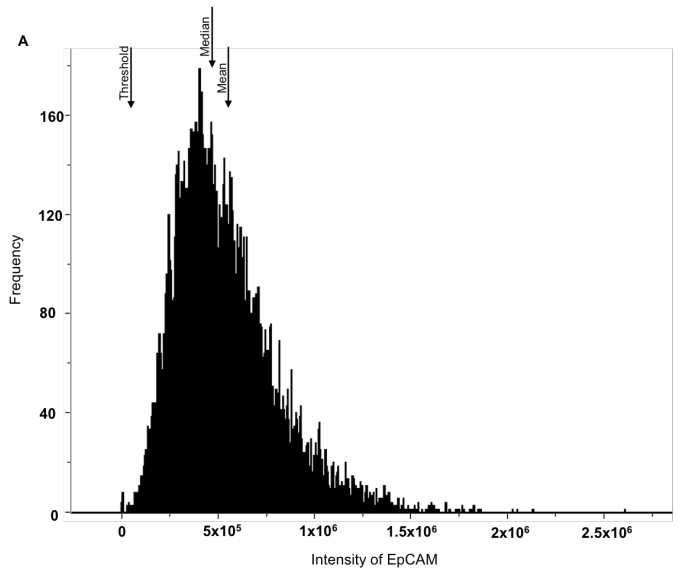
3.2.4 Quantification of fluorescence

The immunofluorescent images captured using the image flow cytometer are easily inspected visually but the Imagestream^x image flow cytometer is also able to collect quantitative information about the immunofluorescence. A number of different methods by which the quantitative fluorescence collected may be analysed were considered (Figure 3.5).

Figure 3.5A shows a histogram of the total fluorescence intensity of the EpCAM immunofluorescence of the 5000 Sk-GT-4 single cells as generated using the IDEAS® software. There is a range of fluorescent intensity demonstrated. We evaluated three possible ways of deriving a single quantitative measure of EpCAM expression by the cells (Figure 3.5B). The simplest involves visual inspection of the cells to determine whether there is immunofluorescence corresponding to EPCAM expression within the cell. Cells at the lower intensities are inspected and a population of positive cells selected as demonstrated by the red line in Figure 3.5A. In these SK-GT-4 cells 99.4% of cells were positive for EpCAM expression based on this arbitrary threshold. This is consistent with the known expression of EpCAM in this positive cell line.

Alternatively it is possible to calculate the median and mean intensity of immunofluorescence across all of the imaged cells. As indicated in Figure 3.5B these correspond to an intensity of 4.9 x 10^5 and 5.4 x 10^5 respectively. These values have

the advantage of being easily comparable between cell lines. Clearly information about the heterogeneity of EpCAM expression will be lost if a single quantitative measure is selected.



B

	Value
Visual threshold	99.4%
Mean intensity	5.4 x 10^5
Median intensity	4.9 x 10^5

Figure 3.5. Methods of quantification of fluorescence captured by image flow cytometry

SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibody against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and incubated subsequently with antibody against EpCAM and with DAPI. Cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at 480-560 nm, EpCAM and 430-505 nm, DAPI. A. A histogram of the total intensity of immunofluorescence between 480 and 560 nm for 5000 individual cells from the EpCAM antibody was generated using the IDEAS® software. B. A threshold for visible EPCAM positivity was defined after inspection of the images (downward pointing arrow). The median and mean intensities of fluorescence of all the cells imaged were calculated (downward pointing arrows).

3.2.5 The effect of masks on quantification of fluorescence

The Imagestream^X image flow cytometer allows localisation of antigens within the cell. During the analysis of the collected images, the IDEAS® software allows discrete areas of the cells to be selected for analysis by application of a mask which delineates a region of the cells, for example the cell membrane or nucleus. Figure 3.6

demonstrates how the application of a mask allows for the measurement of fluorescence in a specific cell location, for example the cell membrane. The masks are created based on the brightfield image of the cell. The mask created is seen overlying the brightfield image (Figure 3.6A). The same mask is shown overlying an image of the immunofluorescence corresponding to EpCAM expression. In this way, only the strong peripheral rim of fluorescence corresponding to the cell membrane will be considered. A problem with the application of this mask is that it does not take account of the original cell having been a three dimensional object (Figure 3.7). Cells are imaged using the image flow cytometer across a plane that runs through the midpoint of the cell (Figure 3.7A). The equivalent two-dimensional image that is captured has a strongly fluorescent outer rim corresponding to the cell membrane at that plane of view (Figure 3.7B). The paler green fluorescence detected within this bright rim corresponds also to membranous EpCAM expression but it is potentially from the membrane that is not on the plane of imaging and hence is not in focus. A mask that only selects the outer rim will underestimate therefore the overall expression of EpCAM for that particular cell. Application of such a mask is only of value if the specificity of the image flow cytometer to detect membrane staining is more important than the sensitivity.

The use of a mask has little impact on the overall shape of the histogram of EpCAM expression (Figures 3.5A and 3.6B). As a result the proportion of cells that are positive for EpCAM expression based on a visual threshold (Figure 3.6B) is also unchanged. The absolute values for the mean and median fluorescence of the cell population are considerably lower when a mask is applied as would be anticipated. The median intensity for example drops from 4.9×10^5 to 8.7×10^4. With patient samples and circulating tumour cells sensitivity may be the more important factor which would mean that there is little role for masks in the analysis of circulating tumour cells.

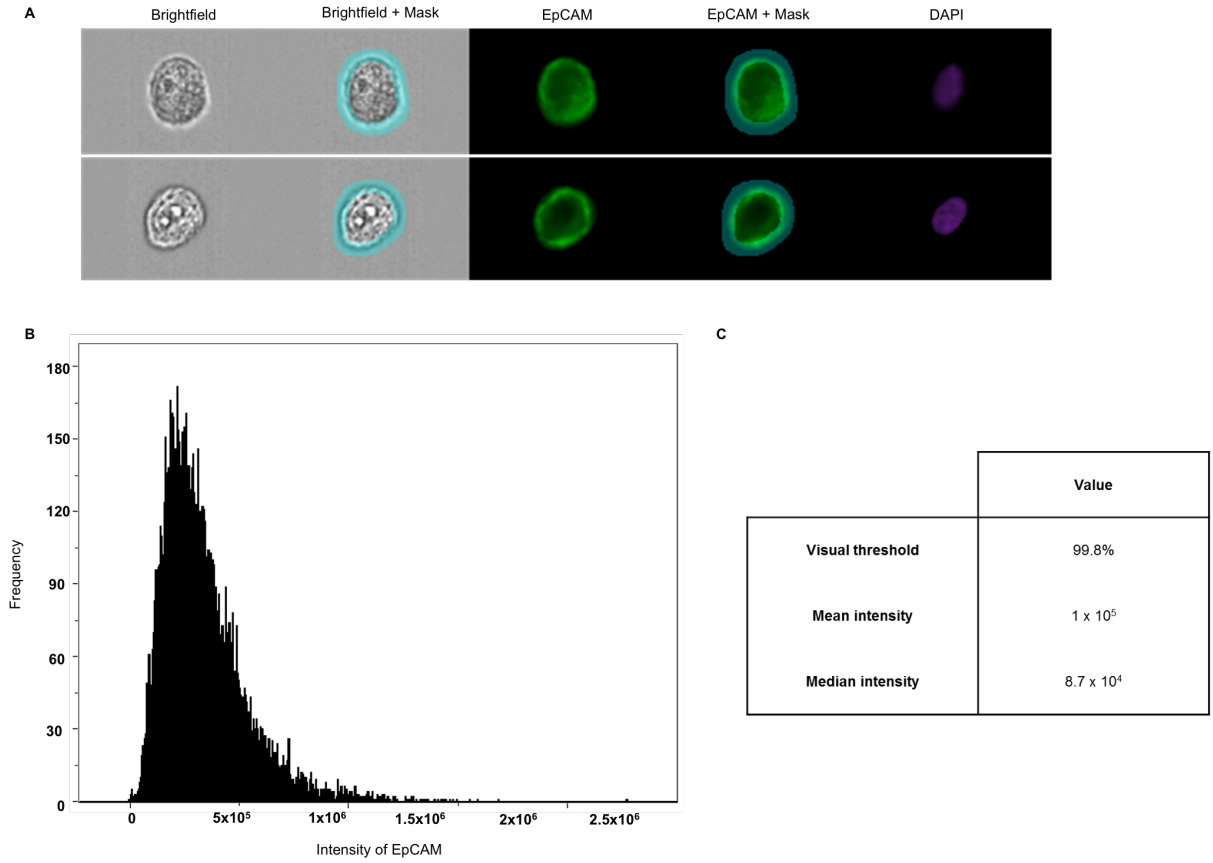


Figure 3.6. The effect of masks on quantification of image flow cytometry fluorescence

SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. Cells were permeabilised by incubation with 0.3 % saponin and incubated with antibody against EpCAM and with DAPI. Cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560 nm, EpCAM and 430-505 nm, DAPI. A. A mask corresponding to the cell membrane was created using IDEAS® based on the brightfield image and is shown over the brightfield image and the EpCAM image adjacent to the same images without the masks for two cells. This mask was then applied to the images of immunofluorescence corresponding to EpCAM. B. A histogram of the intensity of immunofluorescence between 480 and 560 nm within the mask area for individual cells from the EpCAM antibody was generated using the IDEAS® software. C. A threshold for visible EPCAM positivity was defined after inspection of the images. The median and mean intensities of fluorescence of all the cells imaged was calculated.

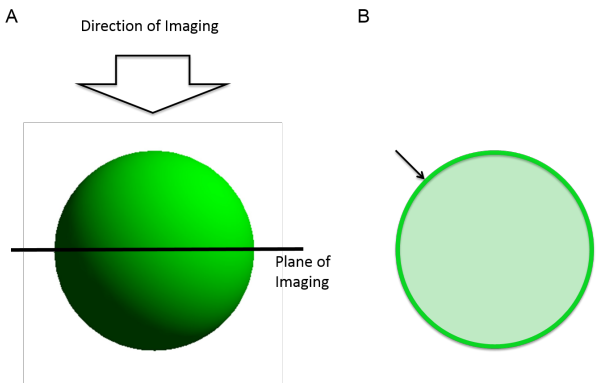


Figure 3.7. The limitation of use of a two dimensional mask when applied to a three dimensional cell.

The small arrow (B) corresponds to the bright rim of fluorescence seen using the image flow cytometer corresponding to the membrane signal.

3.2.6 EPCAM expression in oesophageal cancer cell lines

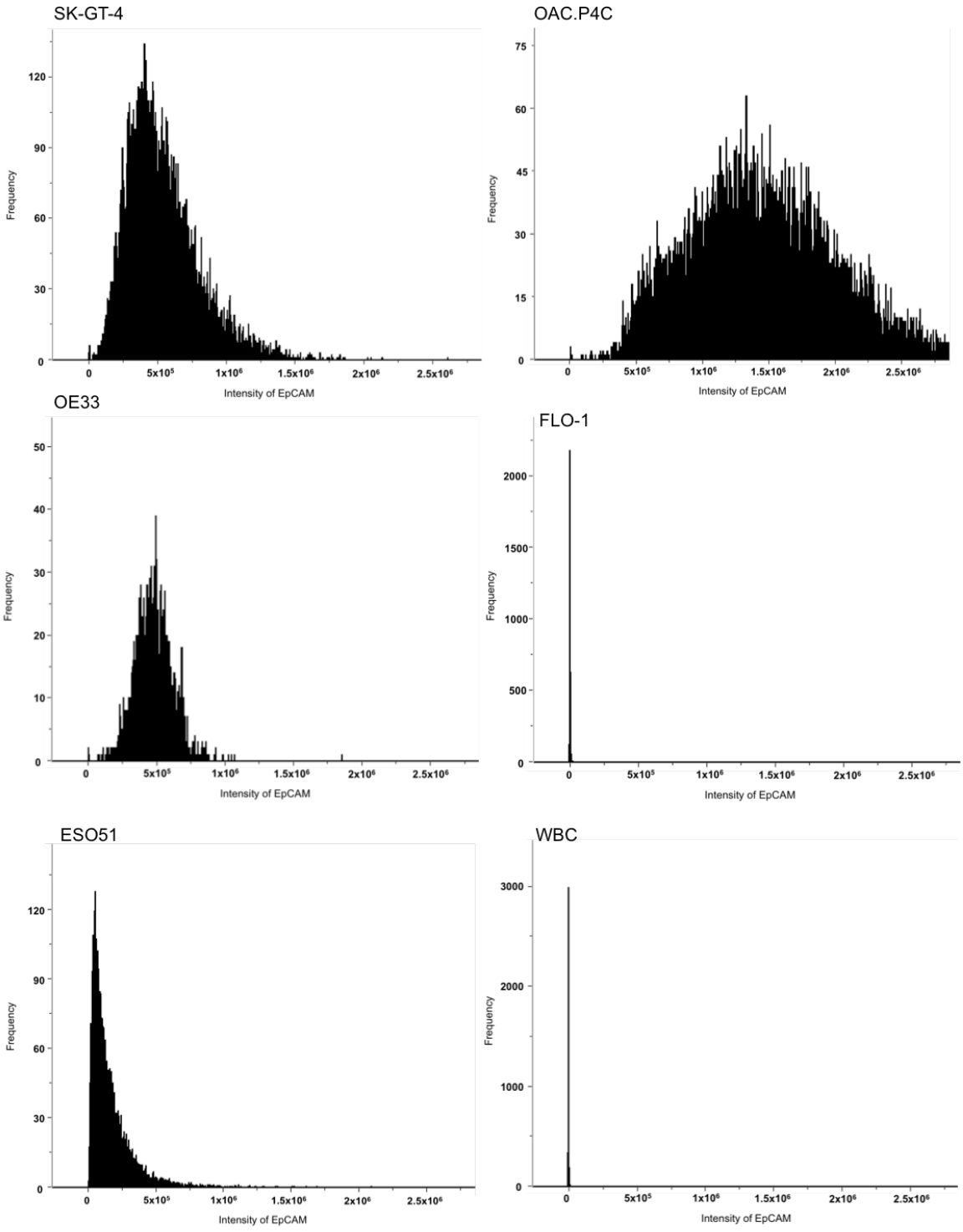
Oesophageal adenocarcinoma is an epithelial derived tumour and EpCAM expression is therefore anticipated in oesophageal adenocarcinoma cell lines. Individual histograms of the frequency of cells against the intensity of EpCAM immunofluorescence are shown for each of five oesophageal adenocarcinoma cell lines (Figure 3.8A). In addition an equivalent histogram for cultured white blood cells is included. The single quantification values of the data are provided (Figure 3.8B).

Four of the five oesophageal cell lines express detectable EpCAM expression. EpCAM expression was not detected in the FLO-1 cells. The absence of EpCAM expression may have occurred in the patient or been lost subsequently during culture. The absence of EpCAM expression in FLO-1 highlights the importance of a detection method for circulating tumour cells that does not solely rely on expression of a single antigen. As expected no EpCAM expression is detected in the white blood cells which indicates that EpCAM expression could be used to distinguish between cancer cells and normal white blood cells.

EpCAM expression was quantified as described in 3.2.4 (Figure 3.8B). This analysis illustrates that an arbitrary threshold of positivity is ineffective for discriminating

between the differences in expression of the oesophageal cancer cell lines. The mean and median values provide a much more effective discrimination and should therefore be chosen when such a quantification comparison is required. OAC.P4C cells, which express higher EPCAM levels, have a much wider variation in the expression of EpCAM between individual cells than the other cell lines.

A



B

	SK-GT-4	OAC.P4C	FLO-1	OE33	ESO 51	WBCs
Visual threshold	99.4%	99%	0%	99.8%	98.7%	2%
Mean intensity	5.4×10^5	1.5×10^6	688	4.8×10^5	1.6×10^5	1.0×10^3
Median intensity	4.9×10^5	1.4×10^6	339	4.8×10^5	1.1×10^5	1.1×10^3

Figure legend on next page

Figure 3.8. EpCAM expression across oesophageal adenocarcinoma cell lines

SK-GT-4, OAC.P4C, FLO-1, OE33, OE19 and cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. ES051 and white blood cells were grown to optimal density in suspension in routine culture medium and 1 x 10^6 cells fixed with 1 % formalin. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibody against EpCAM and with DAPI. Cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560 nm, EpCAM and 430-505 nm, DAPI. A. Following image collection, 5000 single cells were identified and a histogram of the intensity of collection channel 2 corresponding to the conjugated EpCAM antibody was generated for each cell line. B. A threshold for EPCAM positivity was created by visual inspection of the images and in addition the median and mean intensity of fluorescence of the cells imaged was calculated. Statistical analysis was performed with the IDEAS ® software.

3.2.7 Cytokeratin expression in oesophageal cancer cell lines

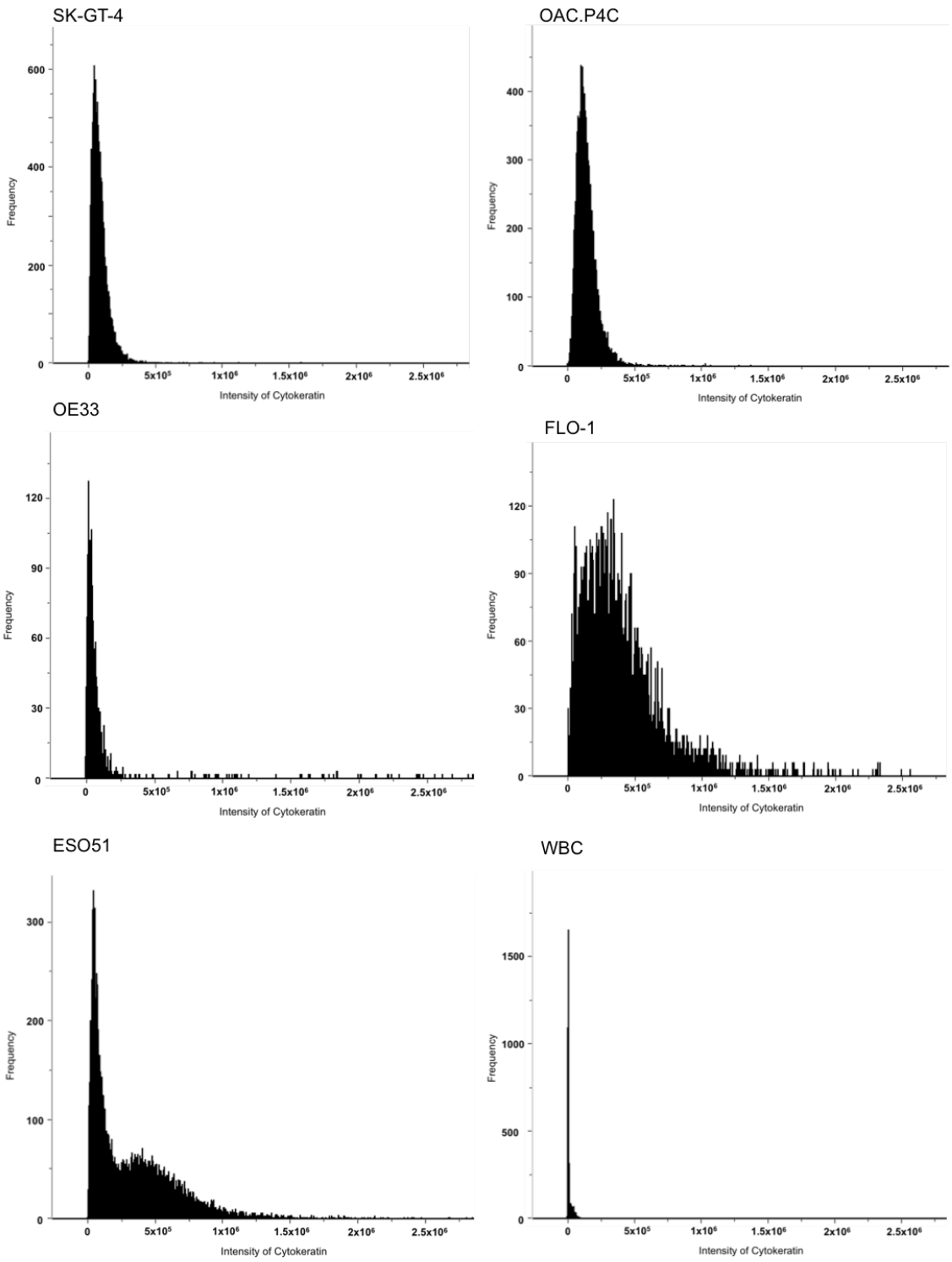
Cytokeratin expression is again expected in all oesophageal adenocarcinoma cells. The expression of epithelial cytokeratins in five oesophageal adenocarcinoma cell lines is shown in Figure 3.9. Individual histograms of the frequency of objects at each intensity of fluorescence corresponding to the cytokeratin expression are shown for each of the five oesophageal adenocarcinoma cell lines and for cultured white blood cells (Figure 3.9A). The single value quantification of cytokeratin expression for the six cell population was calculated from the data (Figure 3.9B).

All of the oesophageal cancer cell lines express epithelial cytokeratins. The white blood cells do not express the cytokeratins which confirms the ability of cytokeratin expression to distinguish circulating tumour cells from normal blood cells. Again, evaluation of the expression based upon the percentage of cells that express more than an arbitrary amount does not effective discrimination between the different expression levels within the different cell lines. The median and mean values represent these differences better and indicate that the level of expression of cytokeratins detected is lower than that of EpCAM in the equivalent cells. (Figures 3.8B and 3.9B).

There appear to be two populations of ESO51 cells with respect to cytokeratin expression. There is a population with low levels of cytokeratin expression and then a second population of cells with higher cytokeratin levels. There is little evidence of

two cell populations for other cell lines. A possible explanation is that the ESO51 cells are cultured in solution and it is therefore more difficult to ensure that all of the cells being analysed are healthy. This contrasts with the adherent cell lines in which unhealthy cells detach and are therefore not collected. It is possible that differences in the viability of cells within the ESO51 population has caused this heterogeneity in cytokeratin expression.

A



B

	SK-GT-4	OAC.P4C	FLO-1	OE33	ESO 51	WBCs
Visual threshold	99.5%	98.9%	98.4%	99.1%	99.9%	13%
Mean intensity	8.7×10^4	1.4×10^5	4.1×10^5	2.8×10^5	3.3×10^5	8.3×10^3
Median intensity	7.1×10^4	1.3×10^5	3.3×10^5	4.4×10^4	2.3×10^5	3.8×10^3

Figure legend on next page

Figure 3.9. Cytokeratin expression in oesophageal adenocarcinoma cell lines

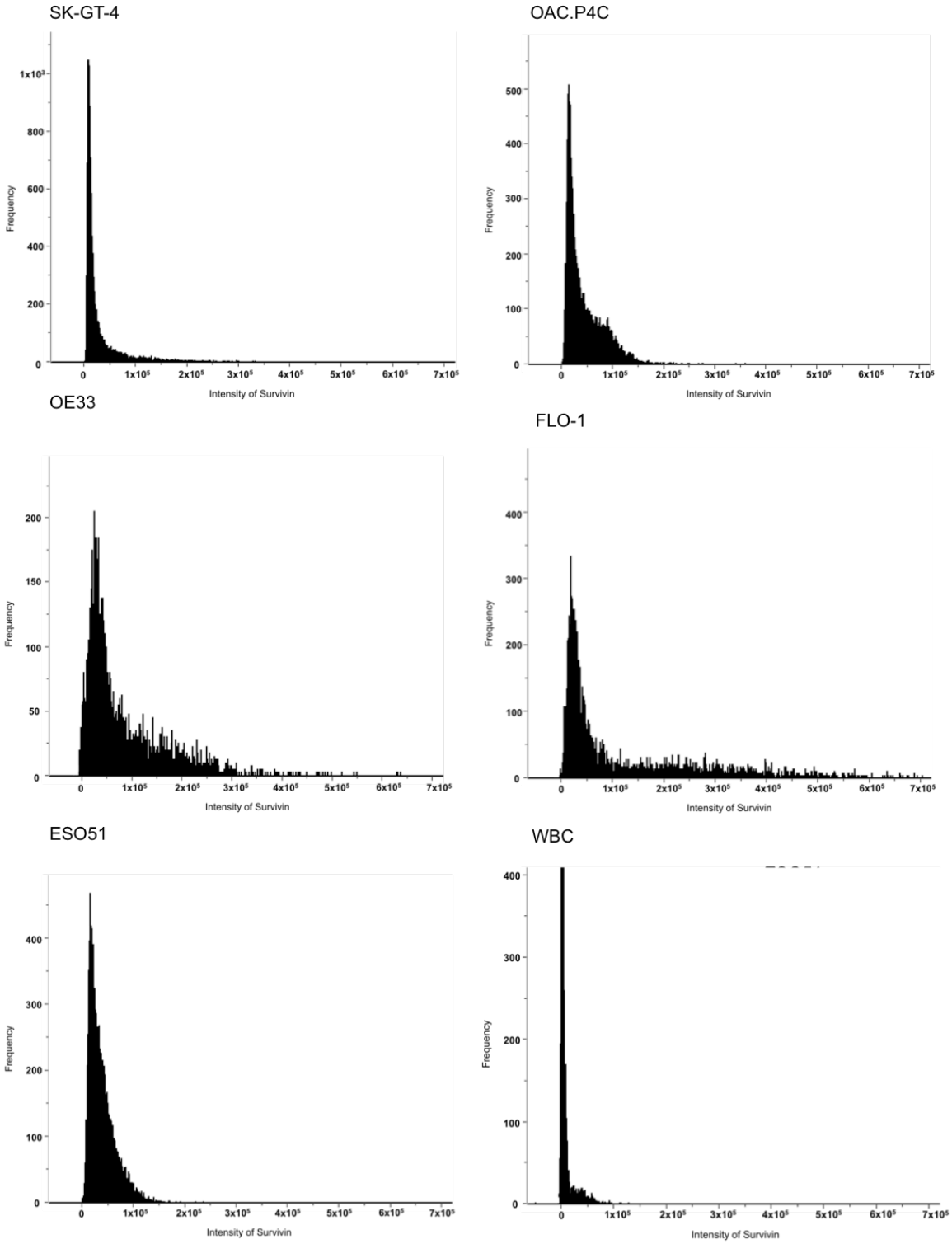
SK-GT-4, OAC.P4C, FLO-1, OE33 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. ES051 and (*need to check with David*) leucocytes were grown to optimal density in suspension in routine culture medium and 1 x 10^6 cells fixed with 1 % formalin. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibody against EpCAM and with DAPI. Cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 560-595 nm, cytokeratins and 430-505 nm, DAPI. A. Following image collection, 5000 single cells were identified and a histogram of the intensity of collection channel 2 corresponding to the conjugated cytokeratin antibody was generated for each cell line. B. A threshold for cytokeratin positivity was created by visual inspection of the images and in addition the median and mean intensity of fluorescence of the cells imaged was calculated. Statistical analysis was performed using the IDEAS ® software.

3.2.8 Survivin expression in oesophageal cancer cell lines

Some survivin expression is detected in all of the oesophageal adenocarcinoma cell lines (Figure 3.10). The overall level of expression detected is lower than for EpCAM and cytokeratin. Survivin has been reported to be found both within the nucleus and at times within the cell cytoplasm. It has been reported that the presence of survivin within the nucleus is associated with increased metastatic potential of the tumour cell.

Survivin is not detected in the white blood cells demonstrating that it may be used to help discriminate oesophageal adenocarcinoma cells from other cells in the blood.

A



B

	SK-GT-4	OAC.P4C	FLO-1	OE33	ESO 51	WBCs
Visual threshold	97.4	95.6	98.7	94.6	97%	7%
Mean intensity	3.2×10^4	4.5×10^4	1.2×10^5	8.8×10^4	3.8×10^4	8.3×10^3
Median intensity	1.5×10^4	3.2×10^4	4.9×10^4	5.4×10^4	3.1×10^4	3.8×10^3

Figure legend on next page

Figure 3.10. Survivin expression in oesophageal adenocarcinoma cell lines

SK-GT-4, OAC.P4C, FLO-1, OE33 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. ES051 and white blood cells were grown to optimal density in suspension in routine culture medium and 1 x 10^6 cells fixed with 1 % formalin. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibody against EpCAM and with DAPI. Cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 560-595 nm, survivin and 430-505nm, DAPI. A. Following image collection, 5000 single cells were identified and a histogram of the intensity of collection channel 2 corresponding to the conjugated cytokeratin antibody was generated for each cell line. B. A threshold for survivin positivity was created by visual inspection of the images and in addition the median and mean intensity of fluorescence of the cells imaged was calculated. Statistical analysis was performed using the IDEAS ® software.

3.3 Discussion

The Imagestream^x image flow cytometer is well suited to the identification and characterisation of malignant cells. The demonstrated ability to distinguish accurately and rapidly malignant cells from white blood cells suggests that image flow cytometry could be used effectively to detect circulating tumour cells in patient blood samples.

The images of tumour cells captured using the Imagestream^x image flow cytometer are of a high quality and allow very accurate characterisation and identification of the cells. Examples of images of oesophageal adenocarcinoma circulating tumour cells imaged using an alternative method are demonstrated in Figure 3.13. The cells were imaged with the Cellsearch® platform (Riethdorf *et al.*, 2007; Bobek *et al.*, 2014). The cells were selected magnetically based on EpCAM expression (Figure 3.13A) or were filtered prior to imaging (Figure 3.13B). The size, shape and antigen expression of the cells can be determined, but it is difficult to be confident that the images detected definitely represent cells and are not debris. Cellsearch® relies on visual inspection to make this distinction. The images captured with the Imagestream^x image flow cytometer are of a higher resolution than those with the CellSearch® platform. This higher resolution improves the confidence with which tumour cells can be identified.

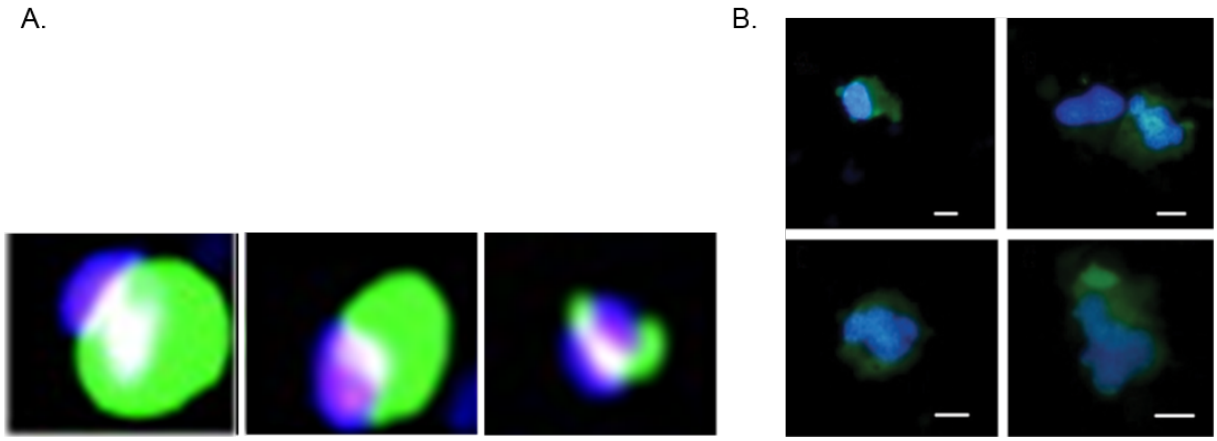


Figure 3.11. Published images of circulating tumour cells detected and imaged using the Cellsearch® platform

A. Oesophageal cells have been labelled with a fluorescently conjugated antibody to cytokeratin (green) and with DAPI (purple) nuclear stain. Images reproduced from A. (Riethdorf et al., 2007), B. (Bobek et al., 2014)

These results demonstrate consistency in the expression of the tumour specific biomarkers across multiple oesophageal adenocarcinoma cell lines whilst they are not identified in white blood cells. This consistency is in keeping with the reported literature on oesophageal adenocarcinoma (Kumble *et al.*, 1996; Kimura *et al.*, 2007; Malhotra *et al.*, 2013) and increases confidence that these biomarkers are likely to be detected in oesophageal adenocarcinoma circulating tumour cells from patient samples. The exception to this is the expression of EpCAM, which is not found in the FLO-1 cell line. The lack of EpCAM expression in FLO-1 cells has been reported (Garcia *et al.*, 2016) and likely represents a lack of EpCAM expression in the original tumour from which the cell line derives. The simultaneous use of multiple biomarkers allows FLO-1 cells, and hopefully all circulating tumour cells without EpCAM expression, to be identified. In contrast, these cells would not be captured by the CellSearch® platform that relies solely on EpCAM expression for the initial separation of circulating tumour cells.

A clear distinction between oesophageal adenocarcinoma cells and white blood cells can be made based upon the expression of tumour specific biomarkers, the absence of CD45 expression and morphology and size. This distinction supports the use of the technique to detect oesophageal adenocarcinoma circulating tumour cells in blood without false positive identification of normal blood cells.

Three different methods of expressing the immunofluorescence detected as a single value using the image flow cytometer have been considered. There is no standardised approach to deciding what level of immunofluorescence is required for an individual cell to be classed as positive. Within cultured cancer cells in which limited heterogeneity might be expected, there is wide variability in the levels of immunofluorescence between individual cells. It is very difficult to therefore build a specific numerical threshold into a definition of what constitutes a circulating tumour cell. Visual inspection of cells therefore remains important although this approach does introduce the potential for a lack of consistency between samples.

The use of cultured oesophageal adenocarcinoma cell lines as a model for circulating tumour cells assumes that the cultured cells express the same antigens and at the same levels patient tumour cells. There is also an assumption that physical properties of the cells such as area will be the same or similar. Optimisation of methods using cultured cells does not therefore fully validate the methods when applied to patient samples.

Chapter 4. Development of a method to enrich circulating tumour cells from whole blood

4.1 Introduction

Cultured cell lines provide useful models with which to develop methods of detection and characterisation of oesophageal adenocarcinoma cells by image flow cytometry, as described in chapter 3. During this validation, high concentrations of around 1 x 10^6 cells per ml were analysed in phosphate buffered saline. This situation is very different from circulating tumour cells which are present in whole blood in concentrations of less than 1 cell per ml (Allard *et al.*, 2004). A method of processing whole blood to achieve a cell suspension containing these rare circulating tumour cells that can then be labelled with antibodies and imaged by image flow cytometry is required. It is advantageous to enrich the circulating tumour cells prior to their analysis. Enrichment can be achieved by positively selecting the circulating tumour cells based upon specific characteristics or by positively depleting the normal blood constituents (Parkinson et al., 2012). The latter method has the advantage of not being reliant upon any single property of the circulating tumour cells, which would mean that only circulating tumour cells with that single property would be analysed. It enables subsequent identification of all circulating tumour cells irrespective of physical characteristics or specific antigen expression.

During enrichment, a proportion of circulating tumour cells will be lost. These losses are quantified by measuring the recovery of circulating tumour cells, defined as the percentage of the original circulating tumour cells in the blood sample that is present after sample enrichment. The higher the purity of circulating tumour cells required, the greater the losses and hence the lower the recovery are likely to be. The great strengths of image flow cytometry are the speed at which large numbers of cells can be imaged and the potential to process samples that still contain relatively large numbers of normal blood constituents. The IDEAS® software allows subsequent identification of the circulating tumour cell population. This capability allows the balance between the circulating tumour cell purification and recovery to be kept in favour of recovery, which minimises the loss of circulating tumour cells.

An enrichment protocol should include methodology to deplete all the different normal cell populations in whole blood. The three main cell populations to be depleted are red blood cells, white blood cells and platelets. Circulating tumour cells are closest, in terms of size and density, to white blood cells. It is from this population that it is most difficult to separate the circulating tumour cells. This represents the major technical challenge in attempting to enrich circulating tumour cells from whole blood.

One millilitre of blood contains up to approximately ten million white blood cells. A number of different methods for depleting white blood cells had been investigated prior to the commencement of this research. Positive selection of the malignant cells involved attempts to identify a universally expressed antigen or differential separation on the basis of cell density or size. Whilst adequate depletion rates of white blood cells could be achieved, the recovery of cancer cells was extremely low. The cancer cells that were recovered were damaged physically as assessed by the images produced (data not shown). In addition, these methods whilst successful in reducing the number of white blood cells created in their place a large quantity of cellular debris. This cellular damage was particularly prevalent with methods based upon passage of blood over columns of magnetic beads. The maximum rate of sample processing by the image flow cytometer is determined by the flow rate of objects that can be either cells or debris. Replacing large numbers of cells with large quantities of cellular debris does not therefore reduce the overall processing time of the sample.

The EasySep™ method of depleting white blood cells has a potential advantage that it should not cause cell damage and hence cellular debris is not produced which makes it very suitable for use alongside the Imagestream^{x} image flow cytometer. EasySep™ CD45 depletion followed by flow cytometry has been reported in the detection of circulating tumour cells in melanoma and colorectal cancer (Fusi *et al.*, 2010; Fusi *et al.*, 2011). In a study focused on squamous cell carcinomas of the head and neck, the EasySep™ magnetic beads were compared with three other commercially-available magnetic beads (Yang *et al.*, 2009). The EasySep™ beads achieved the highest depletion of white blood cells whilst maintaining the highest recovery of circulating tumour cells.

A model of circulating tumour cells in whole blood can be generated by adding cultured oesophageal adenocarcinoma cells into healthy volunteer whole blood. This

model allows evaluation of the impact of the depletion steps on recovery and validation of any overall protocol created.

4.2 Results

4.2.1 Morphology and cross-sectional area of cancer cells in comparison to white blood cells

Distinction between white blood cells and cancer cells can be made on the basis of morphology (Figure 4.1A). The white blood cells are smaller and more rounded with a higher nuclear to cytoplasmic ratio than the cancer cells. The ability to distinguish visually white blood cells form malignant cells provides an additional means by which reassurance that white blood cells will not be mistaken for circulating tumour cells when patient samples are analysed.

The size of cells is another potential method by which to distinguish circulating tumour cells from white blood cells. Examination of the brightfield cell images clearly indicates that OE019 and ESO51 cells are smaller than the other cancer cell lines (Figure 4.1A). Cell areas can be calculated rapidly for large numbers of cells with the Imagestream^{x} image flow cytometer. The cross-sectional area of six oesophageal cancer cell line cells are shown in Figure 4.1. For comparison, an established breast cancer cell line, SK-Br-3, and white blood cells are included. There are significant differences between the mean cross-sectional areas of the six oesophageal cancer cells ($p<0.0001$). The mean cross-sectional area of the breast cancer cell line is within the range of cross-sectional areas of the oesophageal adenocarcinoma cell lines. There does however remain a statistically significant difference between the cross-sectional area of the OE019 cells which are the smallest of the oesophageal cancer cells and the white blood cells ($p<0.0001$). This difference supports the use of cross-sectional area in helping to distinguish circulating tumour cells from white blood cells in patient samples.

Comparison of the nuclear cross-sectional areas of the different cell lines again demonstrates that there are significant differences across the oesophageal adenocarcinoma cell lines ($p<0.0001$). The smallest cells, OE019 and ESO51, not surprisingly have the smallest nuclei (Figure 4.1C). Although visually there is less

difference between the cross-sectional nuclear area of the white blood cells compared with the OE019 cell line, there remains a significant difference between the two ($p<0.0001$). Once again this result suggests a potential role of nuclear cross-sectional area in helping to distinguish circulating tumour cells from white blood cells in patient samples.

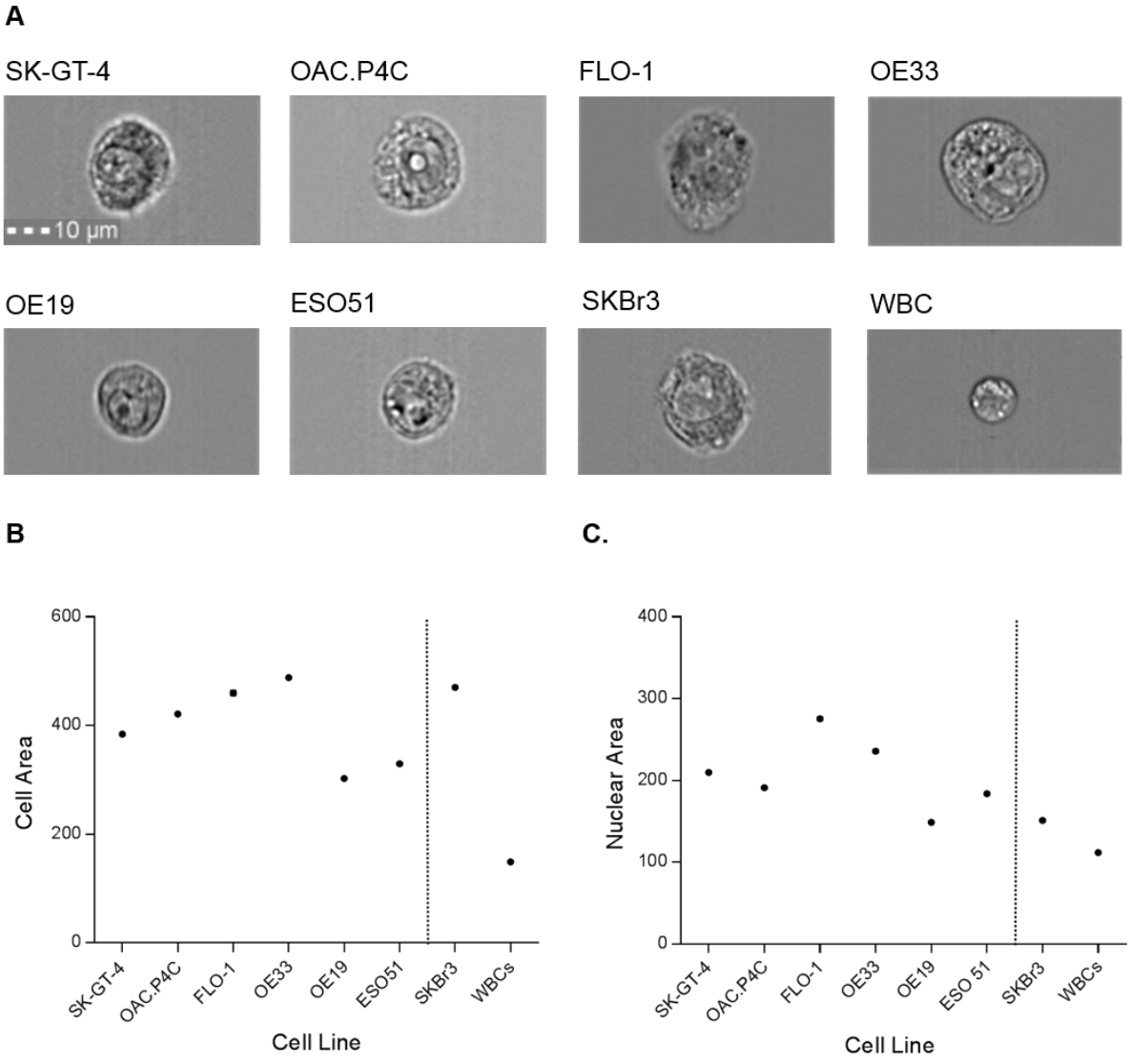


Figure 4.1. Morphology and cross-sectional cell and nuclear areas of cancer cells in comparison to white blood cells

A. SK-GT-4, OAC.P4C, FLO-1, OE33, OE19 and SKBr3 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 4 % formaldehyde. ES051 and white blood cells were grown to optimal density in suspension in routine culture medium and 1 x 10^6 cells fixed with 1 % formalin. Cells were visualised with an Imagestream^X flow cytometer without laser excitation with collection of brightfield images only. B. The cross-sectional cell and nuclear area of 1000 cells from each cell line was calculated using IDEAS®. This data was exported to GraphPad Prism. The mean cross-sectional areas and standard errors of the mean for each cell line are shown An unpaired t-test was used for 2 groups and a one-way ANOVA for multiple groups to compare the mean cell and nuclear cross-sectional areas of the cancer cell lines and white blood cells.

4.2.2 Panel of antibodies for cancer cell detection

Results obtained with the fluorescently-conjugated antibodies previously selected for detection of circulating tumour cells are shown in Figure 4.2. For this validation, cultured SK-GT-4 adenocarcinoma cells were added to whole blood. In the SK-GT-4 cancer cells, EpCAM is located in the cell membrane, cytokeratin is cytoplasmic and survivin is within the nucleus (Figure 4.2A). As expected, there is no fluorescence corresponding to CD45 expression in these cancer cells. CD45 is only expressed in white blood cells and is therefore an excellent biomarker to help distinguish these cell populations.

In addition to expression of CD45, the white blood cells do not demonstrate any fluorescence corresponding to EpCAM, cytokeratin and survivin (Figure 4.2B). As noted above, the white blood cells are small round nucleated cells that could be distinguished from the cancer cells based upon the brightfield images alone. When combined with the fluorescent images, the ease of distinction between white blood cells and cancer cells is illustrated clearly (Figure 4.2C).

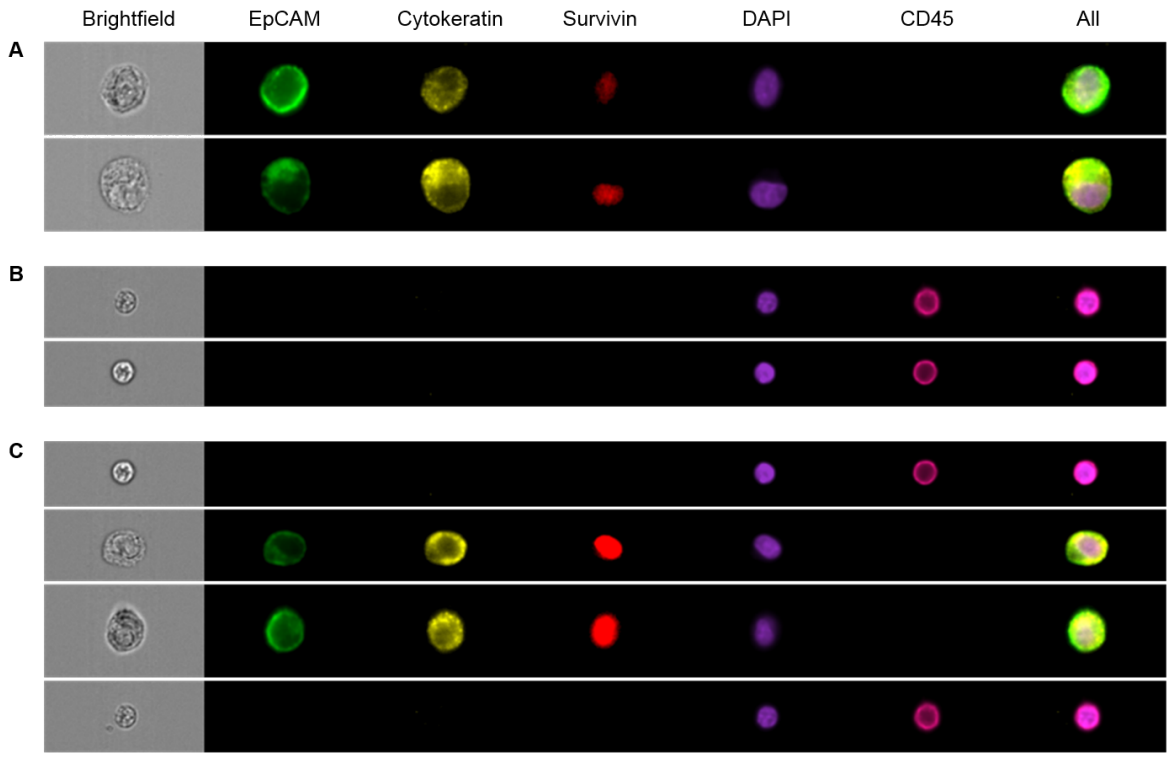


Figure 4.2. Detection of oesophageal adenocarcinoma cells in whole blood by image flow cytometry

A. SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibodies against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and survivin and incubated subsequently with antibody against EpCAM, CD45 and with DAPI. B. Density gradient separation buffer was added to healthy volunteer blood and the sample centrifuged. The fraction of the centrifuged blood sample containing most of the white blood cells (buffy coat) was extracted and fixed with 4% formaldehyde. Cells were permeabilised with 0.3% saponin and incubated with the antibodies as described. All cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560 nm, EpCAM; 560-595 nm, cytokeratins; 642-745 nm, survivin; 745-800 nm, CD45 and 430-505 nm, DAPI.

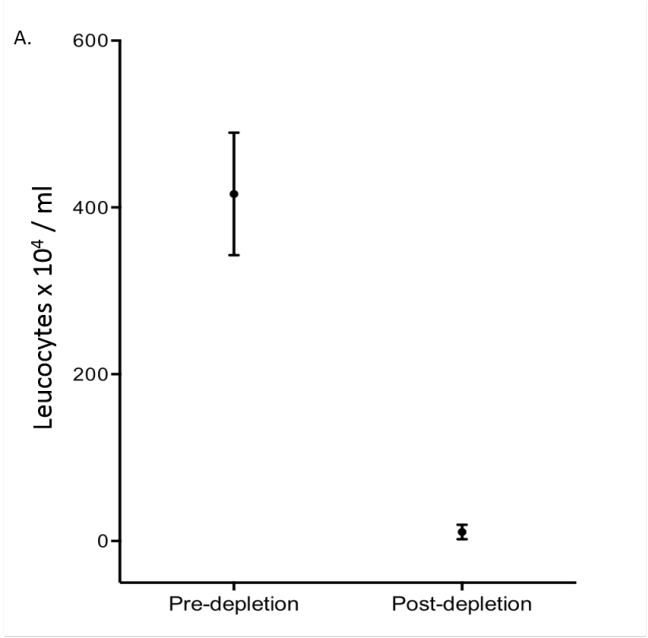
4.2.3 White blood cell depletion

Depletion of white blood cells is an essential process prior to the detection of circulating tumour cells by image flow cytometry. The effectiveness of white blood cell depletion with the EasySep™ method is illustrated in Figure 4.3. Prior to depletion, the mean concentration of white blood cells was 4.16 x 10^6 per millilitre. At

a magnification of x 40 the maximum rate of imaging for the Imagestream^{x} image flow cytometer is 2000 objects per second. Theoretically at this concentration, one millilitre would take approximately 34 minutes to be analysed by the Imagestream^{x} image flow cytometer. There will clearly be a difference in the actual times taken for samples to process reflecting the normal range of white blood cells per millilitre of whole blood of between approximately 3.9 and 10.6 x10^{6} (Chapter 1). This would correspond with a processing time of up to approximately 83 minutes per millilitre of whole blood.

The Figure shows the dramatic reduction in white blood cell concentration achieved with the EasySep™ CD45 enrichment. A percentage depletion of 98% +/- 0.8% was achieved consistently (Figure 4.3A). At the same rate of imaging with the Imagestream^{x} image flow cytometer, the depleted sample will have a theoretical processing time of less than one minute (Figure 4.3B).

It would be possible to place the supernatant from the magnet back in the EasySep™ magnet and repeat the process of white blood cell depletion. This procedure could in theory increase the depletion of white blood cells but at the expense of losing cancer cells and reducing recovery. Given the capability of the Imagestream^{x} image flow cytometer, the purity achieved after placing the sample within the magnet once was considered sufficient to allow sample processing in a realistic time (table 4.3B). Remaining white blood cells within the sample can be distinguished from cancer cells as detailed in section 4.2.4.



B.

Pre-depletion WBC count	Time to process one ml sample (mins)	Post-depletion WBC count	Time to process one ml sample (mins)
4.16×10^6	34	1.07×10^5	0.9

Figure 4.3. Depletion of white blood cells by EasySep™ CD45 depletion.

Five ml whole blood samples from healthy volunteers were fixed in 0.4% formaldehyde for 20 minutes and the red blood cell component was lysed. Platelets were removed with centrifugation. A. White blood cells were counted in a Neubauer haemocytometer. EasySep™ anti-CD45 antibody complex was added to the fixed cells followed by dextran-coated nanoparticles. The suspension was then diluted with Robosep™ buffer and placed in the EasySep™ magnet for ten minutes. The un-retained cell fraction was decanted into a clean tube by inversion of the sample and magnet. Residual white blood cells were counted in a Neubauer haemocytometer. Data are presented as means +/- SEM. B. Based upon imaging objects at 2000 objects per second with the Imagestream^x image flow cytometer, the times required to process a one ml sample before and after white blood cell depletion were calculated.

4.2.4 Imagestream^x IDEAS® software enrichment

Following physical enrichment of the sample as described above (section 4.2.3) a further enrichment was achieved with the IDEAS® software. A compensation matrix

was created based upon samples labelled with a single fluorophore single colour reference samples for each fluorochrome, as described in chapter 3, to allow removal of spectral overlap into adjacent channels from each of the fluorophores used. Figure 4.5 demonstrates the application of the software to identify the tumour cell population.

Cells are identified first based upon the intensity of the nuclear dye retained by the cells (Figure 4.4A). There is a sharp peak around zero that corresponds to Amnis Speedbeads and cellular debris. A second peak at an intensity between 3 and 4 x 10^5 fluorescence units corresponds to single leukocytes. The third peak of intensity between 5 and 8 x 10^5 fluorescence units corresponds to doublets of white blood cells and other cells with a larger nuclear signal than a single leucocyte. This peak is where circulating tumour cells would be found. Objects within this population are selected for further analysis. Any objects to the right of this peak are included to ensure that clusters of cells that may contain circulating tumour cells are identified.

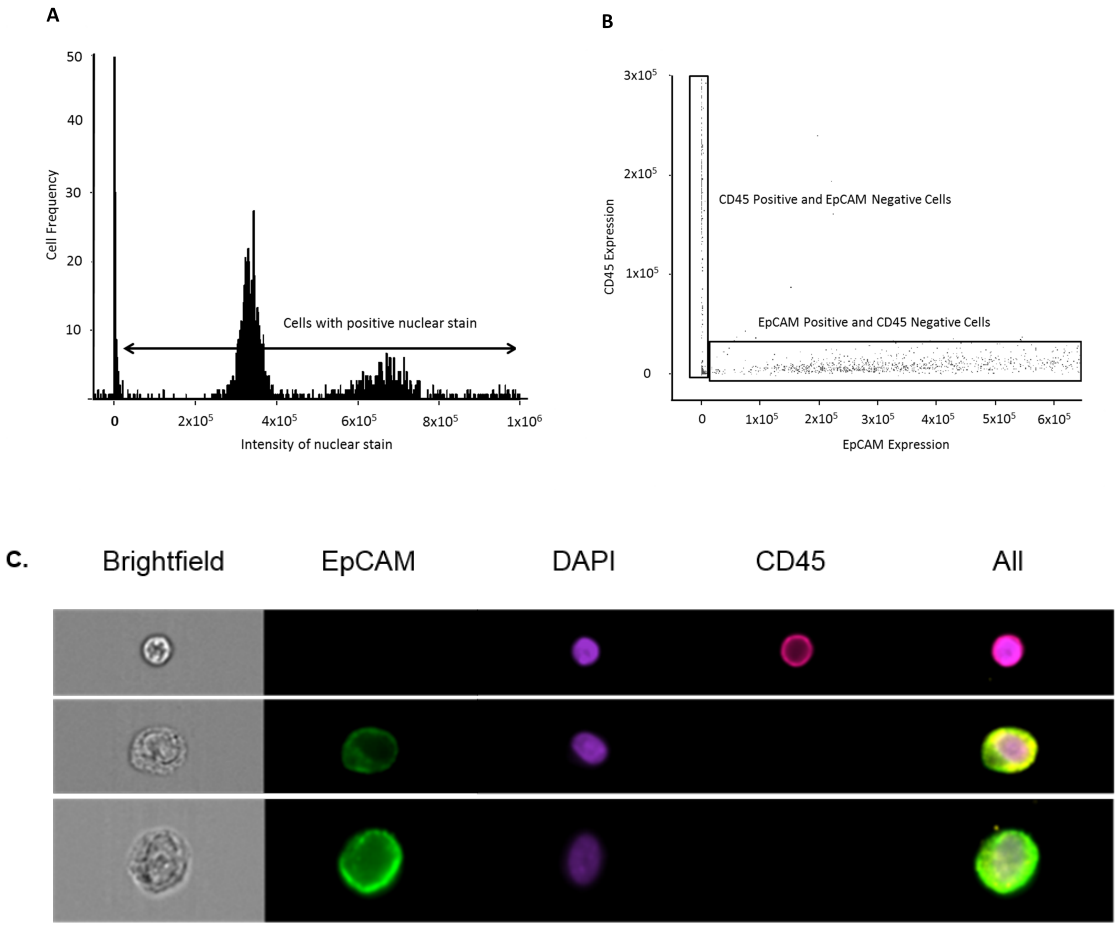


Figure 4.4. Discrimination of the malignant cell population in whole blood from the residual leukocytes after positive depletion of blood cells.

SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. Cells were washed and resuspended in PBS. Appropriate volumes of the prepared cell suspension were added to the healthy volunteer blood sample before any sample processing occurred. After physical sample enrichment, cells were permeabilised by incubation with 0.3 % saponin, incubated with antibodies against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and incubated subsequently with antibody against EpCAM, CD45 and with DAPI. All cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560 nm, EpCAM; 560-595 nm, cytokeratins; 745-800 nm, survivin; 745-800 nm, CD45; 430-505 nm, DAPI. A. A histogram of the intensity of nuclear stain was generated for all objects. The objects were gated to exclude those objects without a nuclear stain. B. Objects with a positive nuclear stain were analysed for the intensity of fluorescence for the CD45 antibody against the intensity of fluorescence of the EpCAM antibody. C. Cells positive for EpCAM and negative for CD45 were then inspected visually. Cancer cells can be distinguished easily from white blood cells by this visual inspection.

Subsequent analysis identifies cells based upon absence of CD45 expression and expression of EpCAM, cytokeratins, and survivin. The effectiveness of the discrimination that can be achieved is demonstrated in Figure 4.4B. A population of cells that express CD45 but not EpCAM is identified clearly and corresponds to the residual white blood cell population. The population of objects that express EpCAM but not CD45 will include the cancer cells. Analysis of the images with IDEAS® Software enables automatic discrimination of the two populations. Similar analysis for survivin and cytokeratin detection allows additional cells to be identified. Any objects that express one or more of the tumour specific antigens and do not express CD45 are selected automatically with the IDEAS® Software. These selected objects are then inspected visually to confirm that the cellular morphology is consistent with a cancer cell.

4.2.5 Losses associated with the Imagestream^x image flow cytometer

The losses associated with the processing of samples with the Imagestream^x image flow cytometer are illustrated in Figure 4.5. These losses can be considered both in terms of the volume of sample lost and the proportion of cells lost. A small volume of each sample is lost during each analysis, This ‘dead space’ should be the same irrespective of the volume of sample processed (Figure 5Ai). The mean volume loss was 5.4 +/- 0.06 µl. As a result, the relative percentage loss of the sample increases as the sample volume decreases (Figure 5Aii). At a sample volume of 20 µl, the loss of volume corresponded to a 26.2% loss of the sample. By contrast at the maximal permitted sample volume of 200 µl, the loss is small at 2.7 %. Analysis of patient samples to detect circulating tumour cells should ideally be with a volume of 200 µl to minimise the impact of these losses.

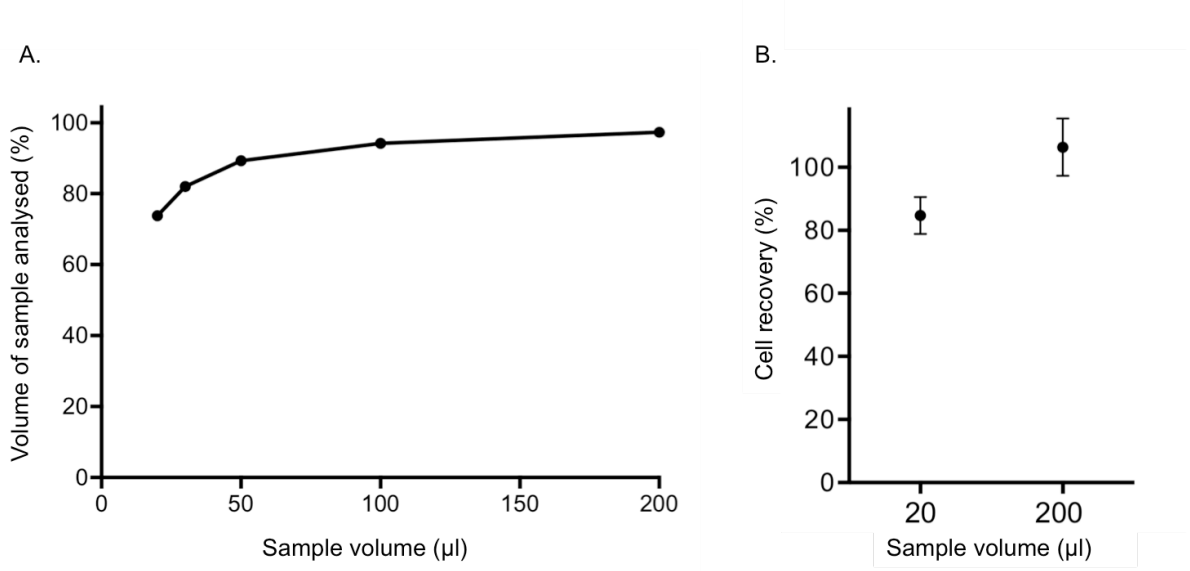


Figure 4.5. The effect of sample volume on sample loss during image flow cytometry.

A. Differing volumes of phosphate buffered saline (from 20 to 200 µl) as measured using a Gilson pipette were analysed on the Imagestream^x without the addition of any cells. The Imagestream^x records the actual volume of the processed sample allowing the percentage of sample lost due to the 'dead space' to be calculated. B. SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. Cells were washed and resuspended in PBS and cell counts were performed using a Neubauer haemocytometer. Samples of 500 cells were resuspended in phosphate buffered saline at a volume of 20 and 200 µl and analysed using the Imagestream^x image flow cytometer. Cells were imaged in the brightfield channel and images with a clear cellular morphology were counted.

The cellular losses that occur during sample processing with the image flow cytometer are illustrated in Figure 4.5B. For a 20 µl sample the mean cellular recovery was 84.7 +/- 5.2 %. For a sample of 200 µl, the mean cellular recovery was 106.4 +/- 8.1%. This value if likely to reflect the margin of error when counting cells using a Neubauer haemocytometer in the context of low cell concentrations. These figures are comparable with the volume losses illustrated in Figure 4.5A which suggests that any cellular losses that occur during sample processing are attributable predominantly to the volume loss. This finding reinforces the importance of analysing patient samples in larger volumes.

4.2.6 Recovery of oesophageal adenocarcinoma cells from whole blood

Reliable detection of circulating tumour cells in patient samples is dependent upon a consistent method of recovery of the tumour cells during purification from the normal blood constituents. The recovery of cells is expressed as the percentage of tumour cells present in a sample that are still present at the end of sample processing. Figure 4.6A shows the recovery achieved after addition of known numbers of cultured oesophageal adenocarcinoma cells to whole blood.

The first attempt at processing whole blood achieved a relatively low recovery of approximately 10% (Figure 4.6A). The final recovery of approximately 50% followed a process of gradual improvement and refinement of the overall method. A clear plateau in the recovery was achieved (Figure 4.6A). It is possible that some of the improvements were as a result of practice. Better technique at each stage of the process would contribute to the improved recovery. The method described has been tested now by other students who have been able to achieve similar recoveries.

Recovery rates at three different cell concentrations were calculated (Figure 4.6B). Consistent recovery was achieved across all cell concentrations and the combined overall recovery was 48.2 +/- 2.4%. Whilst the cell concentrations tested are low, they are close to the concentrations of circulating tumour cells present in patients.

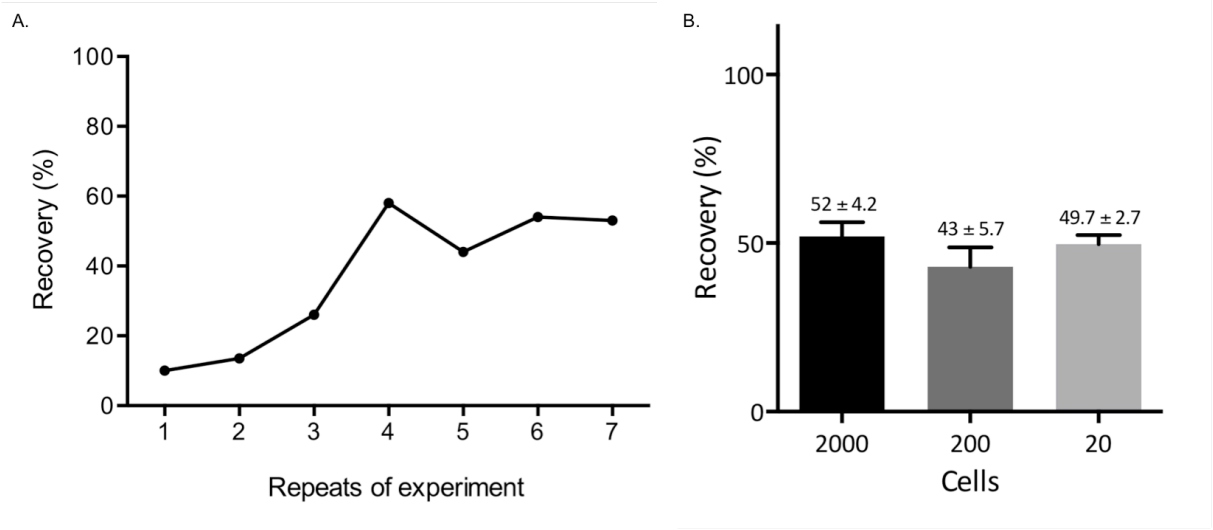


Figure 4.6. Recovery of cultured oesophageal adenocarcinoma cells from whole blood

SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 1 % formalin. Cells were washed and resuspended in PBS and cell counts were performed using a Neubauer haemocytometer. Appropriate volumes of the prepared cell suspension were added to the healthy volunteer blood sample before any sample processing occurred. After sample enrichment, cells were permeabilised by incubation with 0.3 % saponin, incubated with antibodies against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and incubated subsequently with antibody against EpCAM, CD45 and with DAPI. All cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560nm, EpCAM; 560-595 nm, cytokeratins; 745-800nm, CD45; 430-505nm, DAPI. A. Initial cell spiking experiments were performed at a single concentration of 2000 cancer cells in five ml of whole blood. B. Once the full protocol had been developed, further spiking experiments were performed at concentrations of 2000, 200 and 20 cells per 5 ml of whole blood.

4.2.7 Healthy Volunteer Samples

Five samples of blood from healthy volunteers were tested according to the full developed protocol. In none of these samples were any objects identified that would be classified as circulating tumour cells.

4.3 Discussion

Analysing circulating tumour cells without enrichment is achievable with an Imagestream^x image flow cytometer, but the time required to process an un-enriched

sample precludes realistic routine clinical application. My approach to enrichment consisted of two stages. The first was a physical enrichment by depleting normal blood cells. This enrichment was followed by further electronic 'enrichment' of the circulating tumour cells with the IDEAS® software. The method developed for circulating tumour cell enrichment from white blood cells was based on positive depletion of normal blood constituents with no reliance on any single antigen expression by the circulating tumour cells. The method has the potential to be adapted for other tumour types and to characterise the circulating tumour cells detected.

The method developed was demonstrated to achieve a consistent recovery of approximately 48%. A wide variety of recovery rates have been reported in the literature from approximately 11% to 90% (Racila *et al.*, 1998; Benez *et al.*, 1999; Kruger *et al.*, 2000; Liberti *et al.*, 2001). These rates derive from a range of methods of enrichment and detection of the circulating tumour cells, including positive immunomagnetic cell selection, density-gradient centrifugation and filtration. The CellSearch Information for Use documentation produced by Veridex reports recoveries of between 80 – 200% across a range of tumour cell concentrations.

The recovery rate achieved is similar to rates reported with methods based upon in simple positive depletion of normal blood constituents. An overall recovery rate of 46% was achieved with a combination of red blood cell lysis and immunomagnetic depletion of leucocytes in a study of breast cancer cell lines (Lara *et al.*, 2004). It is important to note that not all studies that report recovery rates are comparable. In a study of head and neck squamous cell carcinomas that tested several commercially available anti-CD45 antibodies and magnetic particles including the EasySep™ magnetic beads, a recovery rate of 86% was reported (Yang *et al.*, 2009). The study added cells from cancer cell lines to buffy coat as opposed to whole blood. This difference will lead to higher recovery rates because the losses associated with isolation of the buffy coat are not factored into the recovery calculations.

It is possible to compare the recovery achieved with our method with the theoretical recovery that might be expected based upon the published literature. It is recognised that there are cell losses associated with every centrifugation step. These have been reported as being approximately 7% per centrifugation (Lara *et al.*, 2004). Table 2 summarises the predicted versus observed losses for the method developed

excluding the white blood cell depletion step. This comparison allows an approximate calculation of the losses that occur during the EasySep™ enrichment stage of the protocol. Without accounting for losses associated with the white blood cell depletion the expected recovery would be 60% compared with the observed recovery of 48.2%. The largest contributor to the difference between these two values is likely to be the white blood cell depletion. The one aspect that is not factored into this comparison is the potential losses that occur during the transferring of cells from one tube to another.

Table 4.1 Theoretical losses of recovery at sequential stages of the enrichment and labelling of circulating tumour cells.

Process	Theoretical loss of cells	Residual cell count
Start	0	**100**
RBC lysis	11%*	**89**
Centrifugation	7%*	**83**
Centrifugation	7%*	**77**
(EasySep)	?	**-**
Centrifugation	7%*	**72**
Centrifugation	7%*	**67**
Centrifugation	7%*	**62**
Imagestream[x]	2.7%	**60**

*(Lara *et al.*, 2004)

The wide variations in reported recovery rates between different studies must be taken into account when comparing studies that report circulating tumour cells. As a

result it is virtually impossible to compare or combine studies of circulating tumour cell enumeration. This is one of the major strengths of the CellSearch platform which does allow such comparisons to be made. For an individual method what is important is the reproducibility of the recovery. The method developed achieves this with a consistent recovery across a range of cell concentrations.

Red blood cell depletion can be achieved through density centrifugation or red blood cell lysis. A density gradient medium is added to the whole blood prior to centrifugation. The buffy coat layer generated contains white blood cells and platelets. This layer can be aspirated from the sample for further processing. It is within this buffy coat that circulating tumour cells would be found. Red blood cell lysis relies upon a buffer which is added to whole blood to increase osmotic pressure within the red blood cells which leads to lysis with minimal effect on white blood cells and platelets. Both red cell depletion methods of are simple. There are however significant advantages to red blood cell lysis over density centrifugation with respect to circulating tumour cells. Whilst a high purity of sample can be achieved with density centrifugation, the recovery rate of the buffy coat from the sample is relatively low.

There are inevitably associated losses with red blood cell lysis. It is extremely difficult to quantify accurately these losses using image flow cytometry as it is difficult to process a sample immediately after either lysis to the large number of white blood cells and platelets still present in the sample. The overall recovery achieved by density separation was less than 10% (O'Donnell, 2017). This low recovery is reported in the literature in which much higher losses of circulating tumour cells have been reported after density gradient separation compared with red blood cell lysis (Lara *et al.*, 2004). The 11% loss reported with red blood cell lysis compares favourably with a 27% loss after density gradient separation.

Two red blood cell lysis buffers were tested. Ammonium chloride solution, 0.8 % NH_4Cl, (STEMCELL technologies™) and BD Phosflow™ (BD Biosciences), which is a buffer that contains formaldehyde, diethylene glycol and methanol to lyse and fix cells simultaneously. Both buffers achieved a satisfactory red blood cell lysis. The quality of antibody staining and hence the quality of the final images produced with the Imagestream^x image flow cytometer were felt to be superior after lysis with the BD Phosflow™ compared with the ammonium chloride. There also appeared to be

less cellular debris in the final samples after lysis with BD Phosflow™. Cellular debris is included in the images created by the image flow cytometer and therefore increases significantly the sample processing time. These were subjective measures that were difficult to quantify. Nevertheless, the BD Phosflow™ was selected for the final method.

The size and density of platelets are lower than those of white blood cells and cancer cells. Although platelets and white blood cells are both found within the buffy coat they can be separated easily by differential centrifugation. At a low centrifugal force of 200 g, platelets will remain within the supernatant while white blood cells and cancer cells will be collected in the pellet. A low G centrifugation step was therefore incorporated into the method as detailed in Chapter 2.

Chapter 5. Patient samples

5.1 Introduction

The method developed for enriching whole blood allowed oesophageal adenocarcinoma cells to be identified in an in vitro model (Chapter 4). This method required validation in clinical samples from patients with oesophageal adenocarcinoma patients and from healthy volunteers.

There are a number of different time points during the staging and treatment of oesophageal adenocarcinoma at which circulating tumour cells could be measured. As discussed in Chapter 1, the greatest opportunity to influence treatment decisions in this group of patients is in patients with potentially resectable disease rather than in those with metastatic cancer (section 1.4.2). It was decided therefore to focus on this group of patients.

Many patients with oesophageal adenocarcinoma will undergo peri-opertaive chemotherapy with up to three cycles of neo-adjuvant chemotherapy prior to surgery. This treatment has the potential to affect the number of circulating tumour cells detected. Samples were taken therefore from patients prior to any treatment. The lack of available literature meant that it was not clear whether circulating tumour cells would be detected in this patient group. In patients in whom circulating tumour cells could be identified, the significance of this finding was also unclear. This information could be important potentially in improving the accuracy of staging of oesophageal adenocarcinoma and in designing future studies of circulating tumour cells at different time points during treatment.

5.2 Results

5.2.1 Patient Demographics

A total of 25 patients with oesophageal adenocarcinoma undergoing staging were included in the study (Table 5.1). The majority of patients (n=23) were male and the median age was 68 years (range 58-84). These demographics are in keeping with the overall statistics for age and sex of patients presenting with oesophageal adenocarcinoma.

The clinical and demographic details of the patients included are described in table 1. Patients are ordered according to the full radiological staging of each patient (TNM 7th edition (Figure 1.4)). The study included patients without obvious stage four metastatic disease at the time of blood sampling. All patients had undergone an endoscopy and a computed tomography scan. The complete staging of patients (Figure 1.5) was not completed until after the blood sample had been processed. As a result, the final overall staging revealed that whilst the majority of patients had stage three disease, three patients had stage four, three patients stage two and two patients stage one cancer.

Table 5.1 Patient demographic and staging information.

Patients are ordered according to stage groups (TNM 7th Edition)

Patient No.	Gender	Age (at diagnosis)	Overall Stage (TNM 7th Edition)	Stage Group	Treatment
001	Female	68	T1 N0 M0	1	Surgery
002	Male	82	T1 N0 M0	1	Endoscopic Mucosal Resection
003	Male	83	T3 N0 M0	2	Radiotherapy
004	Female	84	T3 N0 M0	2	Surgery
005	Male	63	T3 N0 M0	2	Chemotherapy + surgery
006	Male	56	T2 N2 M0	3	Chemotherapy + surgery
007	Male	67	T3 N1 M0	3	Chemotherapy + surgery
008	Male	71	T3 N1 M0	3	Chemotherapy + surgery
009	Male	71	T3 N1 M0	3	Chemotherapy + surgery
010	Male	70	T3 N1 M0	3	Chemotherapy + surgery
011	Male	64	T3 N1 M0	3	Chemotherapy + surgery
012	Male	57	T3 N1 M0	3	Chemotherapy + surgery
013	Male	56	T3 N1 M0	3	Chemotherapy + surgery
014	Male	68	T3 N1 M0	3	Surgery
015	Male	67	T3 N1 M0	3	Chemotherapy + surgery
016	Male	75	T3 N2 M0	3	Chemotherapy
017	Male	67	T3 N2 M0	3	Chemotherapy + surgery
018	Male	71	T3 N2 M0	3	Chemotherapy + surgery
019	Male	65	T3 N2 M0	3	Radiotherapy
020	Male	74	T3 N2 M0	3	Chemotherapy + radiotherapy
021	Male	59	T3 N2 M0	3	Chemotherapy + surgery
022	Male	69	T3 N3 M0	3	No treatment
023	Male	63	T3 N3 M1	4	Chemotherapy
024	Male	63	T3 N3 M1	4	Chemotherapy
025	Male	68	T3 N3 M1	4	Radiotherapy

5.2.2 Circulating tumour cell heterogeneity

Examples of the circulating tumour cells identified in this study are shown in Figures 5.1 and 5.2. Composite images of all of the circulating tumour cells identified in a single patient are shown in Figure 5.1. Clear heterogeneity is seen between the individual circulating tumour cells within this single patient sample. This is both in terms of the shape and size of the cells but also in the pattern of fluorescence. A strong nuclear signal is seen in all of the cells however several of the smaller cells

have much lower levels of fluorescence corresponding to the other antibodies used in the panel.

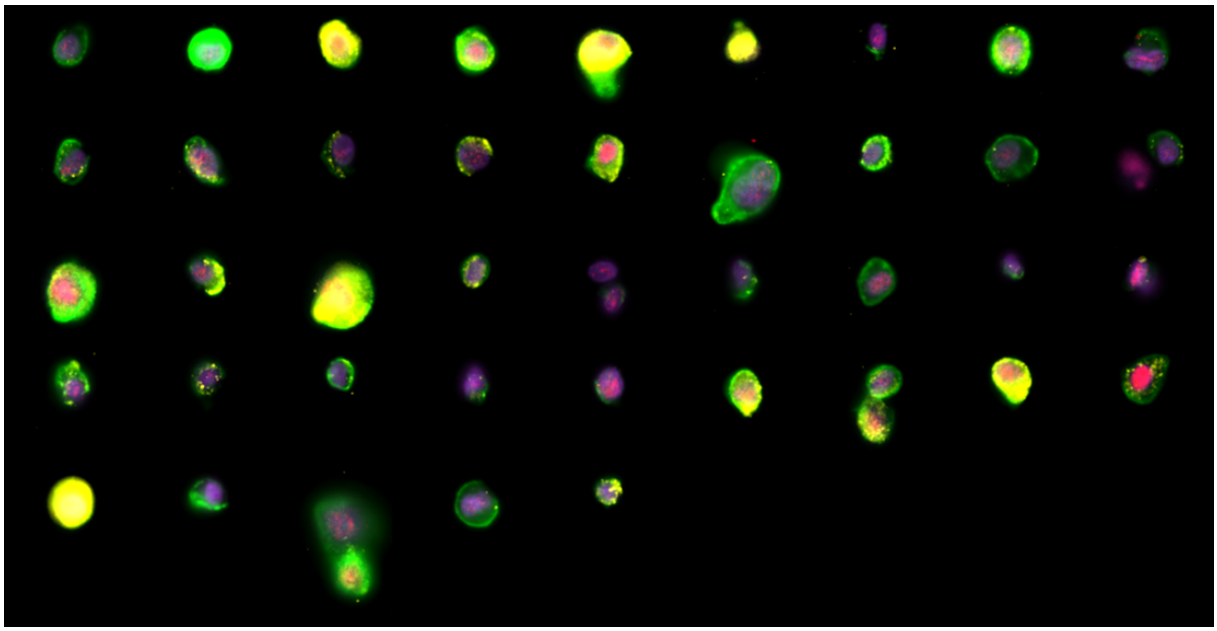


Figure 5.1 Comparison of individual circulating tumour cells from a single patient (patient 006)

Five ml whole blood sample from a patient undergoing staging of oesophageal adenocarcinoma was collected in Transfix (Cytomark, UK) tubes. The red blood cell component was lysed. Platelets were removed with centrifugation at 250 g for 5 minutes. EasySep™ anti-CD45 antibody complex was added to the cell suspension followed by dextran-coated nanoparticles. The suspension was then diluted with Robosep™ buffer and placed in the EasySep™ magnet for ten minutes. The un-retained cell fraction was decanted into a clean tube by inversion of the sample and magnet. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibodies against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and survivin and incubated subsequently with antibody against EpCAM, CD45 and with DAPI. All cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560nm, EpCAM (green); 560-595 nm, cytokeratins (yellow); 642-745nm, surviving (red); 745-800nm, CD45 (pink); 430-505nm, DAPI (purple).

A comparison of representative circulating tumour cells from three patient samples is provided in Figure 5.2 (A-C). Examples of the oesophageal adenocarcinoma cell line SK-GT-4 cells are shown for comparison (Figure 5.2D). In two of the patient samples (A and B), whilst there are circulating tumour cells identified that are very similar to

the SK-GT-4 cells, heterogeneity is also demonstrated within the circulating tumour cell populations. This heterogeneity is again in terms of the size, morphology and antigen expression of the cells.

One of the patient samples tested contained a large population of 85 nucleated cells that were morphologically consistent with a circulating tumour cell in terms of cell shape and size. There was however no fluorescence corresponding to any of the three tumour-specific antibodies (Figure 5.2C). These cells do not appear to be a haematological cell population. It seems likely that these are circulating tumour cells that do not express EpCAm, cytokeratins or survivin. Alternatively they could represent non-epithelial cells within the blood. It has been proposed that metastatic tumour cells may undergo epithelial mesenchymal transition (Willipinski-Stapelfeldt *et al.*, 2005) which provides a possible explanation for the cell population identified. The advantage of the high resolution images captured is that these calls can be identified even though they do not express the three biomarkers.

It is difficult to determine the importance of heterogeneity within a circulating tumour cell population. That all of these cells are identified is a strength of the developed method. For the purposes of circulating tumour cell enumeration all circulating tumour cells are effectively considered to be the identical in terms of their malignant potential. It is possible that some of the differences between the cells relates to cells that are in fact undergoing cell death and hence may have lost their characteristic antigen expression.

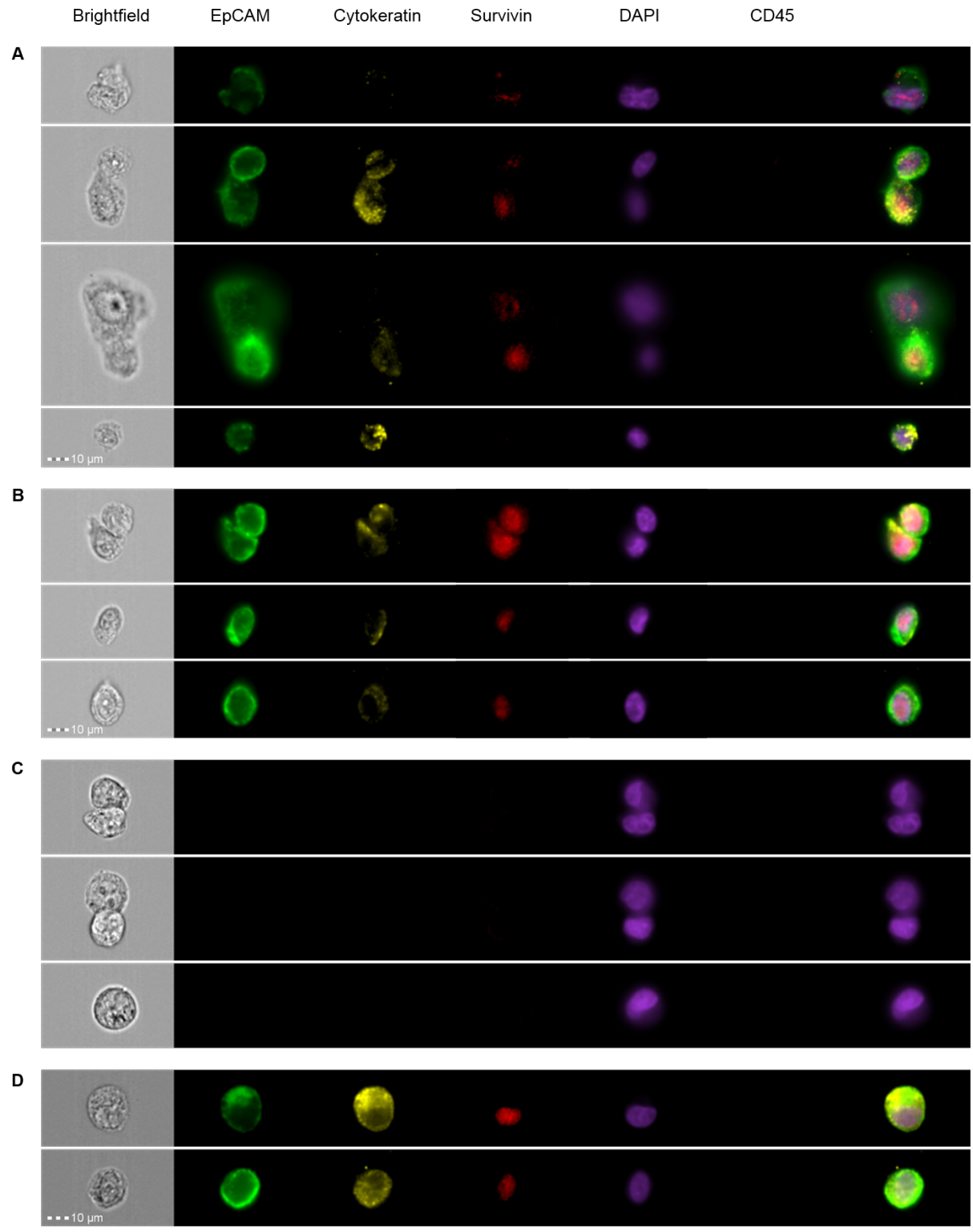


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Figure 5.2 Circulating tumour cells detected in patients with oesophageal adenocarcinoma

A-C. Five ml whole blood samples from patients undergoing staging of oesophageal adenocarcinoma were collected in Transfix (Cytomark, UK) tubes. The red blood cell component was lysed. Platelets were removed with centrifugation at 250 g for 5 minutes. EasySep™ anti-CD45 antibody complex was added to cell suspension followed by dextran-coated nanoparticles. The suspension was then diluted with Robosep™ buffer and placed in the EasySep™ magnet for ten minutes. The un-retained cell fraction was decanted into a clean tube by inversion of the sample and magnet. D. SK-GT-4 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 4 % formalin. A-C. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibodies against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and survivin and incubated subsequently with antibody against EpCAM, CD45 and with DAPI. All cells were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560 nm, EpCAM; 560-595 nm, cytokeratins; 642-745 nm, survivin; 745-800 nm, CD45; 430-505 nm, DAPI.

5.2.3 Circulating tumour cell area

The mean area of the circulating tumour cells detected in the patient samples is comparable with that of cultured oesophageal cancer cell lines (Figure 5.3A). A similar correlation exists is observed with the mean nuclear area (Figure 5.3B). The detected circulating tumour cells are closest in size to the OE19 and ESO51 cell lines. Only the patients with larger numbers of cells were included in this comparison due to the need for meaningful averages. The larger standard error of the mean for both these samples may reflect the greater heterogeneity in size within patient samples compared with cultured cell lines. It may also reflect the smaller number of cells compared with the cultured cell lines. The mean nuclear and cell sizes of the circulating tumour cells is significantly greater than that of white blood cells ($p<0.0001$) which is consistent with the findings for the cultured cell lines. The heterogeneity in circulating tumour cell size described above indicates that dependence upon the size of cells for isolation may not be useful to distinguish circulating tumour cells from white blood cells. The cellular area of one of the smallest circulating tumour cells detected was 164.5 units which is close to the mean cellular area of the white blood cells of 149.5 units. Reliance upon size alone might therefore lead to this cell not being included in the circulating tumour cell population.

It is important to incorporate both antigen expression, size of cells and morphological appearance of the cells when defining the circulating tumour cell population.

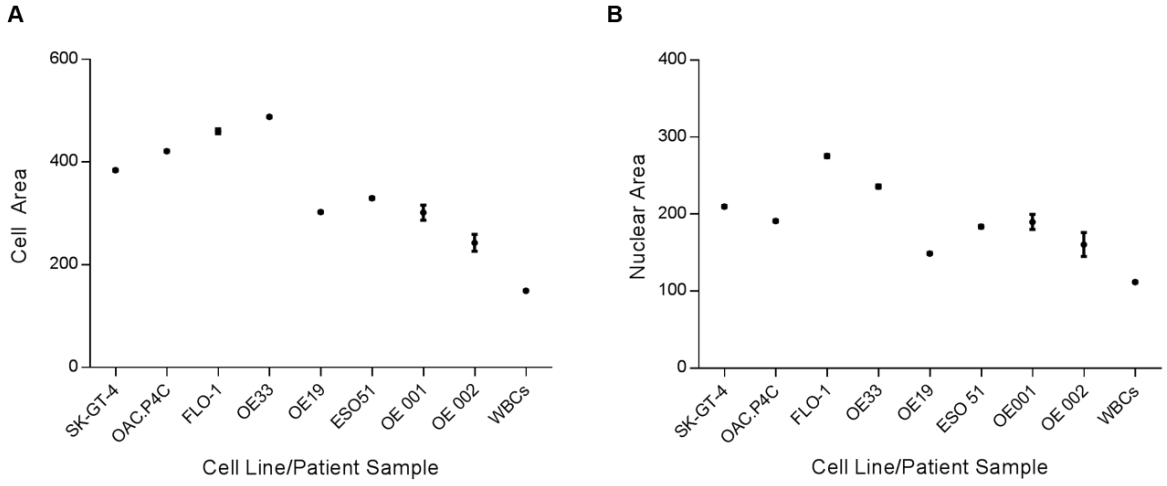

Figure 5.3 Size of circulating tumour cells detected in patient samples compared with oesophageal adenocarcinoma cell lines

A and B. SK-GT-4, OAC.P4C, FLO-1, OE33, OE19 cells were grown to 80 % confluence in routine culture medium, trypsinised and 1 x 10^6 cells fixed with 4% formalin. ES051 and white blood cells were grown to optimal density in suspension in routine culture medium and 1 x 10^6 cells fixed with 1 % formalin. Cells were visualised with an Imagestream^X flow cytometer without laser excitation with brightfield collection only with a x40 objective. Five ml whole blood samples from patients undergoing staging of oesophageal adenocarcinoma were collected in Transfix (Cytomark, UK) tubes. The red blood cell component was lysed. Platelets were removed with centrifugation at 250 g for 5 minutes. EasySep™ anti-CD45 antibody complex was added to cell suspension followed by dextran-coated nanoparticles. The suspension was then diluted with Robosep™ buffer and placed in the EasySep™ magnet for ten minutes. The un-retained cell fraction was decanted into a clean tube by inversion of the sample and magnet. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibodies against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and survivin and incubated subsequently with antibody against EpCAM, CD45 and with DAPI. All cells were visualised with an Imagestream^X flow cytometer. The cell and nuclear areas of 1000 cells from each cell line and of all of the circulating tumour cells from the two patient samples (001 and 002) was calculated with IDEAS®. These data were exported to GraphPad Prism and the mean area and standard error of the mean for each cell line are shown.

5.2.4 Additional objects identified from patient samples

In addition to healthy white blood cells and circulating tumour cells, a number of other populations of objects were detected when patient samples are analysed. Whilst some of these are clearly cellular in nature, some are harder to identify. Inaccurate identification of these objects as circulating tumour cells is a potential source of error when interpreting the results of studies of circulating tumour cells.

In all patient samples a population of what morphologically appear to be white blood cells was identified. These cells were positive for all of the fluorescently-conjugated antibodies and the nuclear stain. (Figure 5.4A). Morphologically the white blood cells appeared darker than other white blood cells with a denser appearance to the cell. These cells may represent a population of apoptotic white blood cells. Alternatively they could simply be white blood cells that have been damaged during sample processing. During method development and validation, these cells were detected in blood samples in to which cultured oesophageal adenocarcinoma cells had been added suggesting that their presence is not related to underlying malignancy in the patient. Whilst these individual cells can be distinguished easily from circulating tumour cells, the discrimination may be less straight forward when these white blood cells are found in clusters (Figure 5.4B). Because the cells are in clusters, the false impression of a larger cell that appears to express the antigens associated with a tumour cell is given. However these cells are all positive for CD45, which provides a simple means to discriminate them from circulating tumour cells.

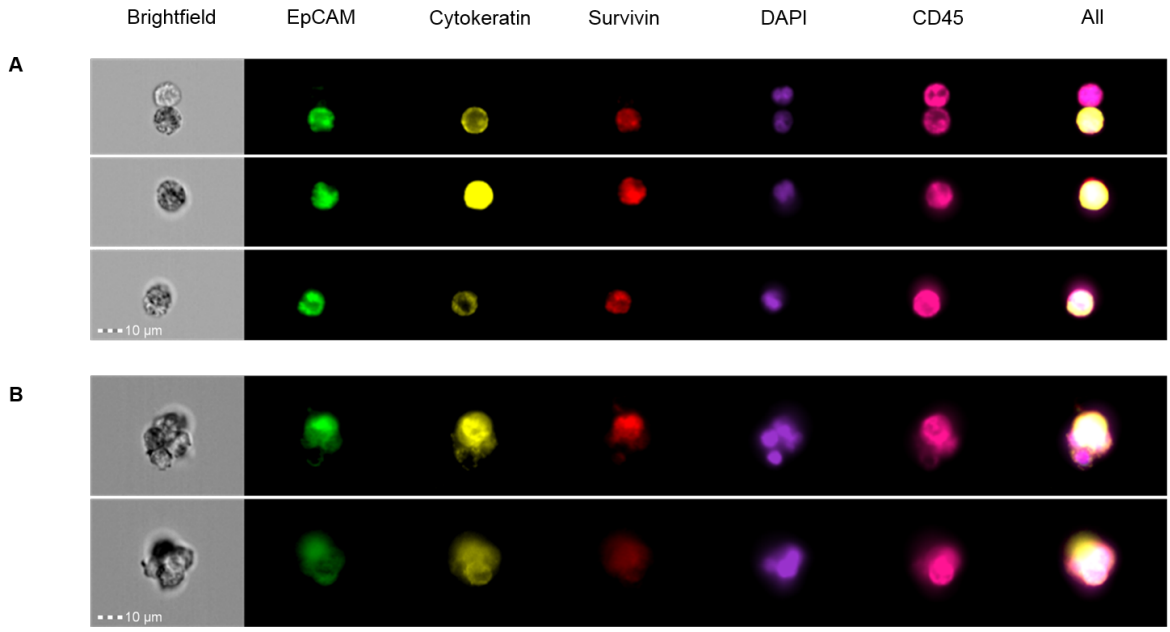

Figure 5.4 Fluorescently-labelled white blood cell population identified during processing of patient samples

Five ml whole blood samples from patients undergoing staging of oesophageal adenocarcinoma were collected in Transfix (Cytomark, UK) tubes. The red blood cell component was lysed. Platelets were removed with centrifugation. EasySep™ anti-CD45 antibody complex was added to cell suspension followed by dextran-coated nanoparticles. The suspension was then diluted with Robosep™ buffer and placed in the EasySep™ magnet for ten minutes. The un-retained cell fraction was decanted into a clean tube by inversion of the sample and magnet. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibodies against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and survivin and incubated subsequently with antibody against EpCAM, CD45 and with DAPI. All cells/objects were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560nm, EpCAM; 560-595 nm, cytokeratins; 642-745nm, survivin; 745-800nm, CD45; 430-505nm, DAPI.

The processing of the blood prior to analysis may create cellular debris. Debris is present as irregular objects of differing size that frequently have a uniform fluorescence across the whole object. An example of a commonly identified population of such objects is shown in Figure 5.5. These objects appear morphologically to be rather flat two dimensional sheets and are significantly larger than either circulating tumour cells or white blood cells. The fluorescence corresponding to the tumour specific antibodies and nuclear stain is uniform across

the entire object. Commonly these objects do not show any fluorescence corresponding to the antibody against CD45. These objects do not appear to be cellular however it is possible, given the lack of CD45 labelling, that they represent debris from tumour cells.

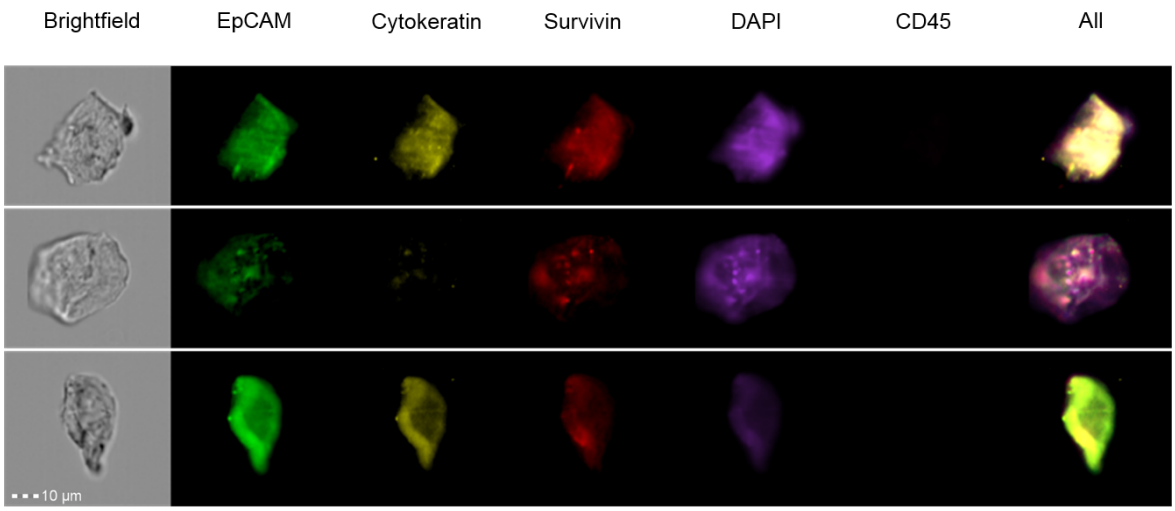

Figure 5.5 Fluorescently-labelled debris identified during processing of patient samples

Five ml whole blood samples from patients undergoing staging of oesophageal adenocarcinoma were collected in Transfix (Cytomark, UK) tubes. The red blood cell component was lysed. Platelets were removed with centrifugation. EasySep™ anti-CD45 antibody complex was added to cell suspension followed by dextran-coated nanoparticles. The suspension was then diluted with Robosep™ buffer and placed in the EasySep™ magnet for ten minutes. The un-retained cell fraction was decanted into a clean tube by inversion of the sample and magnet. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibodies against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and survivin and incubated subsequently with antibody against EpCAM, CD45 and with DAPI. All cells/objects were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560nm, EpCAM; 560-595 nm, cytokeratins; 642-745nm, survivin; 745-800nm, CD45; 430-505nm, DAPI.

An unusual population of cellular objects was detected in large numbers in single patient sample (Figure 5.6). These objects appear to be often multinucleated with either an oblong or spindle morphology. In one of the objects there was evidence of survivin expression but there was otherwise no positive detection with any of the

panel of antibodies used. These objects do not look like circulating tumour cells. A possible explanation is that these objects may represent circulating tumour-associated macrophages. Tumour-associated macrophages are specialised differentiated macrophages that can be found within most tumours (Heusinkveld and van der Burg, 2011). A study of patients with breast, prostate and pancreatic cancer identified a population of large circulating cells thought to represent disseminated tumour-associated macrophages. (Adams *et al.*, 2014). It has been proposed that these giant macrophages may be a prognostic biomarker for some solid tumours. The images of the giant macrophages reported are very similar to those collected in the patient with oesophageal adenocarcinoma (Figure 5.6).

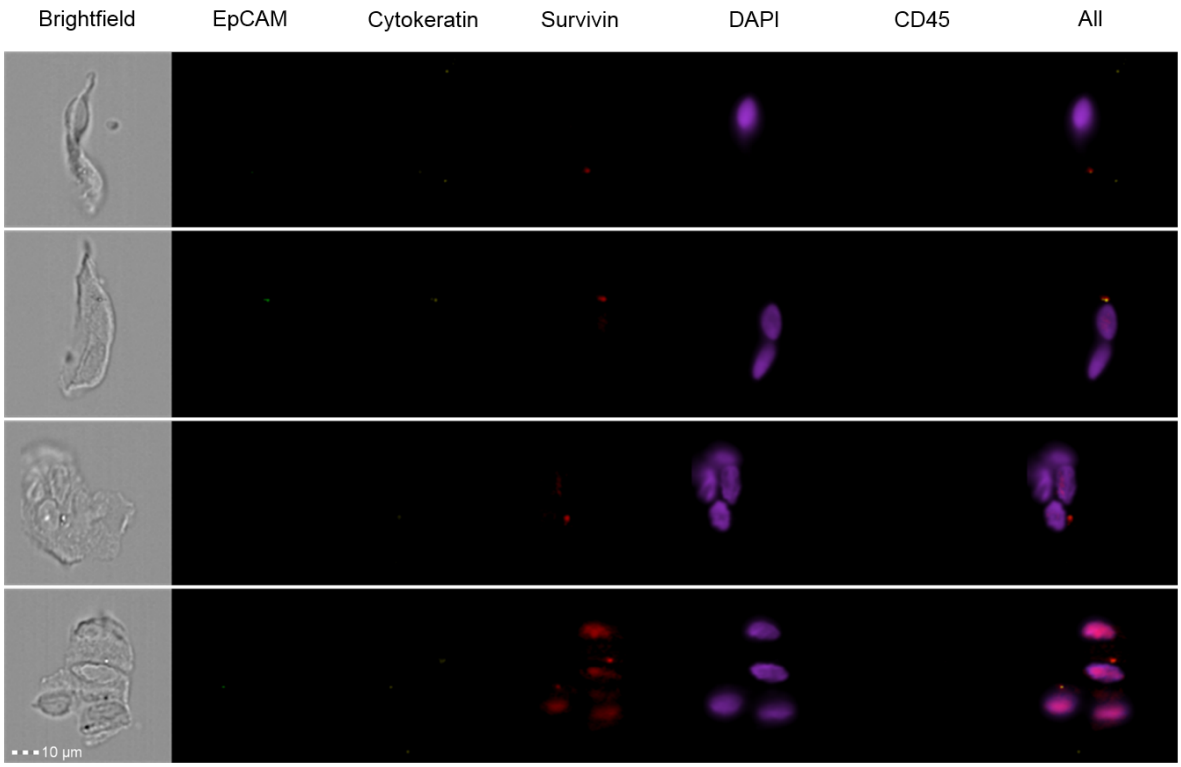

Figure 5.6 Circulating tumour-associated macrophages identified in patient samples

Five ml whole blood samples from patients undergoing staging of oesophageal adenocarcinoma were collected in Transfix (Cytomark, UK) tubes. The red blood cell component was lysed. Platelets were removed with centrifugation. EasySep™ anti-CD45 antibody complex was added to cell suspension followed by dextran-coated nanoparticles. The suspension was then diluted with Robosep™ buffer and placed in the EasySep™ magnet for ten minutes. The un-retained cell fraction was decanted into a clean tube by inversion of the sample and magnet. Cells were permeabilised by incubation with 0.3 % saponin, incubated with antibodies against cytokeratins 4, 5, 6, 8, 10, 13, and 18 and survivin and incubated subsequently with antibody against EpCAM, CD45 and with DAPI. All cells/objects were visualised with an Imagestream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm. Images were collected with a x40 objective with the wavelengths for the collection channels set at: 480-560nm, EpCAM; 560-595 nm, cytokeratins; 642-745nm, survivin; 745-800nm, CD45; 430-505nm, DAPI.

5.2.5 Patient Outcomes

The number of circulating tumour cells detected in each patient sample is listed in Table 5.2. Circulating tumour cells were not detected in the majority of patients. In five cases, circulating tumour cells were identified. Four of these patients had at least stage 3 disease. In two samples only two circulating tumour cells were identified. Three samples had a relatively high number of circulating tumour cells, 85, 43, 17

and cells respectively. This corresponds to 128, 64 and 27 cells per 7.5 millilitre of blood (volume as measured by CellSearch platform). The highest number of cells was identified in the patient sample in which the circulating tumour cells had no fluorescence corresponding to the tumour specific antigens used. These cell numbers are comparable with those reported in other tumour types for patients with metastatic disease (Allard *et al.*, 2004).

Table 5.2 Circulating tumour cell numbers and outcomes for patients included in the study

Patient No.	Stage Group	Circulating tumour cells detected (per 5 ml of blood)	Treatment	Duration of follow up (months)	Follow up status
001	1	0	Surgery	47	Alive disease free
002	1	0	Endoscopic Mucosal Resection	45	Alive disease free
003	2	0	Radiotherapy	8	Died disease recurrence
004	2	0	Surgery	16	Died disease recurrence
005	2	2	Chemotherapy + surgery	28	Died disease recurrence
006	3	43	Chemotherapy + surgery	47	Alive disease free
007	3	17	Chemotherapy + surgery	16	Died disease recurrence
008	3	0	Chemotherapy + surgery	4	Died (post-operative complications)
009	3	85	Chemotherapy + surgery	35	Died disease recurrence
010	3	0	Chemotherapy + surgery	42	Alive disease free
011	3	0	Chemotherapy + surgery	16	Died disease recurrence
012	3	0	Chemotherapy + surgery	41	Alive disease free
013	3	0	Chemotherapy + surgery	25	Alive with recurrence
014	3	0	Surgery	10	Died disease recurrence
015	3	0	Chemotherapy + surgery	18	Alive disease free
016	3	0	Chemotherapy	14	Died of disease
017	3	0	Chemotherapy + surgery	10	Died of Disease
018	3	0	Chemotherapy + surgery	45	Alive disease free
019	3	0	Radiotherapy	5	Died of disease
020	3	0	Chemotherapy + radiotherapy	43	Alive disease free
021	3	0	Chemotherapy + surgery	40	Alive disease free
022	3	0*	No treatment	24	Died of disease
023	4	2	Chemotherapy	9	Died of disease
024	4	0	Chemotherapy	5	Died of disease
025	4	0	Radiotherapy	4	Died

*Tumour-associated macrophages identified (Section 5.2.4)

The overall survival of patients included in the study from time of diagnosis is illustrated in Figure 5.4. The median overall survival for all patients included in the study is 35% with a two year survival of 52%. For those patients who underwent neo-adjuvant chemotherapy and surgery the median survival was again 35% with a 2

year survival of 66%. In this subgroup of patients the pattern of disease recurrence was distant metastatic in 5 patients and locoregional in 3 patients.

The median follow up for the patients sampled in the study is 18 months. All follow up for patients who have undergone oesophagectomy is based on clinical assessment rather than radiological or endoscopic imaging. The latest follow up status of the patients included in the study is detailed in table 2.

The small number of patients with positive circulating tumour cells makes interpreting their relevance to overall survival difficult. Four of the patients with detectable circulating tumour cells underwent surgery and peri-operative chemotherapy. The remaining patient underwent palliative chemotherapy alone. Of the four patients undergoing treatment with curative intent, three died of disease recurrence at 16, 28 and 35 months. All had distant metastatic disease at the time of recurrence. The remaining patient was alive without disease recurrence at 47 months (Table 2). The presence of circulating tumour cells did not correlate with a worse overall survival in those patients undergoing treatment with curative intent (Figure 5.5B)

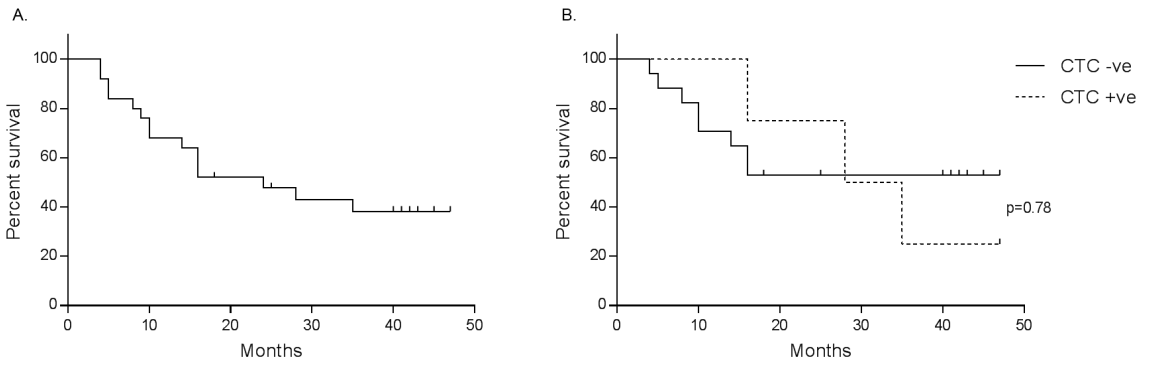

Figure 5.7 Survival of patients included in study

Kaplan-Meier plot of the overall survival of all patients (A.) included in the study and those patients who underwent treatment with curative intent (B.). Overall survival was calculated from date of diagnosis to death or follow up. Data was plotted using GraphPad Prism.

5.3 Discussion

These results from patient samples demonstrate that circulating tumour cells can be detected in patients with oesophageal adenocarcinoma who have no evidence of distant metastatic disease by traditional staging modalities. In this patient cohort, only five out of 25 patients were found to have detectable circulating tumour cells. It is not possible to draw strong conclusions about the impact of these circulating tumour cells on clinical outcomes. There is equally little available literature with which to compare this finding. Only one study has reported the detection of circulating tumour cells in patients with non-metastatic oesophageal adenocarcinoma prior to undergoing surgical treatment. Cells were isolated based upon size using a filtration method (Bobek *et al.*, 2014). This study included 14 patients and identified circulating tumour cells in 78.6%.

It is very difficult to eliminate all subjectivity from the detection of circulating tumour cells. Where there is subjectivity there is the potential for error. Where there was doubt over whether objects did represent circulating tumour cells, the objects were reviewed independently by another researcher. The problem of subjectivity is not limited to our study. Many available methods for circulating tumour cell detection including the CellSearch® platform rely on trained operators who distinguish the circulating tumour cells from other non-cellular objects.

One of the challenges highlighted through the analysis of these patient samples is distinguishing circulating tumour cells from other cellular or non-cellular debris. The high quality images captured with the Imagestream^{x} image flow cytometer are invaluable to this analysis. Some of the circulating tumour cells detected with this method are very similar in both morphology and antigen expression to the established oesophageal adenocarcinoma cell lines used during the development and validation of the method. Circulating tumour cells that display considerable heterogeneity are also detected. The ability to detect all circulating tumour cells even in the presence of heterogeneity is a clear advantage of the method developed and helps to improve the accuracy of the study.

Chapter 6. Concluding Discussion

6.1 Summary of work

This thesis describes the development, validation and application of a novel method for detection of circulating tumour cells in patients with oesophageal adenocarcinoma. This method captures high resolution images of the individual circulating tumour cells. These cells can be enumerated accurately but the method has also the potential for tumour cell biology to be studied. Importantly, the method developed can be modified to allow for the detection of a number of different tumour cell types (Dent *et al.*, 2016).

Blood samples from patients undergoing staging for oesophageal adenocarcinoma prior to treatment were tested for the presence of circulating tumour cells. None of the patients included in the study had evidence of distant metastatic disease based upon radiological staging by computed tomography scan. Where detected, these cells were compared with commercial oesophageal adenocarcinoma cell lines. A clinical role for circulating tumour cells in oesophageal adenocarcinoma is yet to be determined. This research and described method provides a platform for future studies about a potential role of circulating tumour cells at different stages of oesophageal adenocarcinoma treatment.

6.2 Patient cohort

The majority of clinical studies about the role of circulating tumour cells have focused on patients with metastatic disease (Allard *et al.*, 2004; Cristofanilli *et al.*, 2004; de Bono *et al.*, 2008). The overall tumour burden is greater than in patients without metastatic spread. It is far more likely that circulating tumour cells will be detected in oesophageal adenocarcinoma patients with metastatic disease than in those without. These patients represent a logical cohort in which to study circulating tumour cells because it is known that the cancer has already demonstrated ability to metastasise.

This study focused deliberately on patients without known metastatic disease. A much lower detection rate of circulating tumour cells than might have been achieved had metastatic patients been included was anticipated and realised. The lower

detection rate of circulating tumour cells is a potential criticism of this work. It is difficult to draw firm conclusions about the relevance of the presence of circulating tumour cells at the time of diagnosis on patient outcomes. Whilst one of the patients with the highest number of circulating tumour cells did develop metastatic disease early after surgery, a second patient with circulating tumour cells was alive without evidence of disease recurrence 47 months from diagnosis.

It is patients without metastatic disease who are most frequently encountered within my personal clinical practice. It is within this patient group that detection of circulating tumour cells could have the potential to influence the treatments patients will undergo as discussed in Chapter 1 (Section 1.4.2.). Little is known about the relevance of circulating tumour cells in this patient group. It has been proposed that haematological metastasis is found at a very early stage of cancer development (Klein, 2008). With a sensitive method for detection, circulating tumour cells might be found in the majority of patients with cancer irrespective of stage. At the outset of the study there was no information about the frequency with which circulating tumour cells could be detected in patients with oesophageal adenocarcinoma without distant metastases. A single study of circulating tumour cells in patients with gastrointestinal tumours included ten patients with non-metastatic oesophageal squamous cell carcinoma (Hiraiwa *et al.*, 2008). A mean number of only 0.1 +/- 0.3 circulating tumour cells were found in these patients using the Cellsearch® platform. Oesophageal squamous cell carcinoma and oesophageal adenocarcinoma are however two distinct diseases (Siewert and Ott, 2007) and these results are not transferable to patients with oesophageal adenocarcinoma.

Metastatic oesophageal cancer has a dismal prognosis. Therapeutic options are limited with palliative chemotherapy only able to offer a very modest survival benefit (Grunberger *et al.*, 2007). The enumeration of circulating tumour cells in this patient group offers only modest benefit to patients. The survival differences are modest in studies of cancers that have demonstrated a correlation between circulating tumour cells and prognosis (Cristofanilli *et al.*, 2004; de Bono *et al.*, 2008). A difference in overall survival of approximately 8 months was reported between patients with metastatic breast cancer stratified on the basis of detection of circulating tumour cells (Cristofanilli *et al.*, 2004). These studies rarely lead to alterations in therapy. Measuring circulating tumour cells is expensive and time consuming. To have a widespread role in the modern NHS it must do more than it currently offers. It is likely

that future studies of circulating tumour cells in patients with metastatic cancer will focus more on cell biology and response to therapy as opposed to simple enumeration (Sacanna *et al.*, 2011; Baccelli *et al.*, 2013). In this regard the method developed during this study is well suited to future applications.

6.3 Cohort size

The number of patients included within this study was designed to allow validation of the method developed and to test whether circulating tumour cells could be identified in patients with oesophageal adenocarcinoma without radiological evidence of metastatic disease. A common criticism of studies linking circulating tumour cell levels to survival is that they do not provide adequate adjustment for confounding factors. In the context of oesophagectomy for example, factors such as post-operative complication and even surgeon experience have been reported to influence oncological survival (Luc *et al.*, 2015) (Booka *et al.*, 2015) (Markar *et al.*, 2016). To adequately adjust for all such variables would require a larger cohort of patients and be beyond the scope of this study.

Measuring circulating tumour cells involves a single blood sample being taken at a single point in time. The volume of blood sampled at any one time is small, representing approximately 0.1% of the circulating blood volume. It is highly likely that the number of circulating tumour cells present in the bloodstream varies with time and potentially circulating tumour cells could be found in all patients with cancer at some stage. It is possible that circulating tumour cells would be detected in more patients if multiple samples were taken at different time points. There is an area of potential for future studies. It would be interesting to study the effect of treatments including neo-adjuvant chemotherapy and then surgery itself on the levels of circulating tumour cells found in patients.

6.4 Biomarker selection

There is no single oesophageal adenocarcinoma specific biomarker upon which to base the detection of circulating tumour cells. For other tumour types such as prostate and ovarian cancer, there is a clinically applicable biomarker, prostate

specific antigen (PSA) and cancer antigen 125 (CA125), respectively. In this study a combined panel of EpCAM, cytokeratin and survivin was used. All three antigens are not expressed by normal blood cells.

One difficulty in detecting circulating tumour cells is the heterogeneity in antigen expression. Defining the criteria for positively identifying circulating tumour cells must take this heterogeneity into account. A large number of different methods of detection and hence definitions of circulating tumour cells have been described (Parkinson *et al.*, 2012). Whilst some cells may express all chosen antigens, others may only express a single antigen. The fewer antigens expressed the more difficult it is to confidently identify a cell as being a circulating tumour cell. It is a difficult balancing act between capturing all heterogeneous circulating tumour cells and not accidently including non-cancerous objects. In this study, if doubt existed as to whether an object was a circulating tumour cell it was discounted. This may have resulted in a lower rate of circulating tumour cell detection. There were objects identified that looked morphologically like cells but did not express any antigens (Chapter 5). It is highly likely that these are circulating tumour cells but care must be taken when making this assumption. One limitation of the technique developed is that once imaged, objects are not retained and therefore cannot be subjected to any further downstream analysis. Future studies may include filtration methods for detecting circulating tumour cells that may allow for further analysis.

During the processing of samples cellular debris is generated. The method we chose for white blood cell enrichment avoided passage of the blood sample over columns and reduced the debris produced. When processing cells added to blood and patient samples, the cellular debris was detected by some of the antibodies against tumour-specific antigens (Chapter 5). It is likely that this interaction with the antibody is non-specific in the majority of cases although it is possible that some of the cellular debris is derived from tumour cells as has been suggested by others. (Allard *et al.*, 2004).

6.5 Host response

The host immune system plays an important role in tumour dissemination. In oesophageal adenocarcinoma surgery, post-operative septic complications have been reported to impact negatively on oncological outcomes (Lerut *et al.*, 2009).

Post-operative blood transfusion has a similar negative impact on long term outcomes (Dresner *et al.*, 2000), thought to be due to the immune suppression caused by transfusion (Takemura *et al.*, 2003). The ability of circulating tumour cells to cause metastases involves interplay between the tumour cells and the host immune system. The role of the host response is not addressed in the majority of circulating tumour cell research. There is emerging evidence of the importance of this interaction, and the role that suppression of the host immune response plays in the dissemination of circulating tumour cells (Mohme *et al.*, 2017).

One advantage of the method developed in this thesis is that it could be applied to study this immune response. Whilst the work described in the developed protocol sought to remove as many white blood cells as possible a significant number are still processed and imaged. The only labelled antibody used to positively identify the white blood cells was against CD45. Additional antibodies could be added to the panel to study specific markers within the white blood cell population.

6.6 Limitations of circulating tumour cell research

There are a large number of reported techniques for the detection of circulating tumour cells (Parkinson *et al.*, 2012). There are several commercially available platforms in addition to published methods such as that described in this thesis. It is likely that a single blood sample would yield a different number of circulating tumour cells if processed with different methods. If clinical decisions rely on single circulating tumour cells the difference in results obtained with differing techniques may be important. There is a lack of data providing comparisons between these differing techniques. Where such comparisons exist there are inevitably differences found in the number of circulating tumour cells detected by the different methods (Gervasoni *et al.*, 2011; Hofman *et al.*, 2011). Such a large number of described methods undermines clinical confidence in circulating tumour cells. To become widely accepted as a clinical tool there needs to be a greater understanding of the strengths and weaknesses of each approach.

A criticism of circulating tumour cell enumeration is the assumption that all circulating tumour cells are identical. Studies that classify patients into prognostic groups based upon the number of circulating tumour cells rely on the hypothesis that every

circulating tumour cell has the same malignant potential. This may be an oversimplification. The majority of circulating tumour cells are likely to undergo cell death within the bloodstream and not lead to metastases, with the half-life for circulating tumour cells suggested to be as short as one or two hours (Meng *et al.*, 2004). An alternative clinical application of circulating tumour cell research may then be to focus on the cell biology of the tumour cells. In breast cancer, correlations have been reported between the cytokine CXCR4 expression within tumour cells and the development of metastases in bone (Sacanna *et al.*, 2011). In oesophageal adenocarcinoma, CXCR4 expression in the primary tumour has been reported to be associated with the development of micrometastasis to lymph nodes and bone marrow (Kaifi *et al.*, 2005). There is growing interest in the importance of HER 2 expression and amplification in oesophageal adenocarcinoma cells and alternative chemotherapy regimens that may be used in HER-2 positive patients (Smyth *et al.*, 2016). The method developed in this study is ideal for research of this type. The panel of antibodies used in the detection of the circulating tumour cells could be expanded or modified to include antibodies against CXCR4 or HER 2, for example.

There is equally debate as to the exact origin of circulating tumour cells. They are often described as originating from the primary tumour itself. Alternatively, they may be released from pre-existent metastatic or micro-metastatic disease**.** In breast cancer, circulating tumour cells have been detected in patients up to 22 years after treatment with curative intent (Meng *et al.*, 2004). This late development of metastatic disease implies that a balance may exist between tumour cell replication and cell death within micrometastatic disease for many years after apparent curative treatment. It has been proposed that haematological spread of tumour occurs much earlier in the process of tumour development and growth than has been accepted previously (Klein, 2008). Circulating tumour cells may therefore be a very important population of cells to study to fully understand the process of cancer dissemination.

There is clearly a difficulty in determining whether an individual circulating tumour cell has the potential to establish a secondary metastatic tumour. An alternative approach for research looking at the process of metastatic dissemination might be those cells that have already demonstrated this malignant potential. In oesophageal adenocarcinoma multiple lymph nodes are resected along with the primary tumour. Cancer cells found within these nodes are by definition metastatic cells that have spread via lymphatic drainage of the tumour. An interesting direction for future

studies would be to compare circulating tumour cells with tumour cells detected in metastatic lymph nodes. Analysis of primary tumour tissue and potentially tissue from distant metastatic sites could be included. Previous studies have reported that disseminated tumour cells in the bone marrow of patients with oesophageal adenocarcinoma have prognostic significance (Thorban *et al.*, 2000; Vashist *et al.*, 2012). A short section of rib which is removed routinely at oesophagectomy to allow better access into the thorax, may be a better source of bone marrow than traditional iliac crest sampling (Mattioli *et al.*, 2001)**.** This routine sampling of the rib allows the metastatic cells within the bone barrow to be studied. It may be that a valuable role of circulating tumour cells is in helping to understand the process of tumour growth and dissemination.

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Appendix A. Patient consent form

The Newcastle Upon Tyne Hospitals NHS
NHS Foundation Trust

Use of Diagnostic Samples and Samples Taken During Therapy to Support Research into Diseases and Cancer – to be stored in the Newcastle Cancer Centre Biobank

INFORMATION SHEET

Why have I been approached?
You will shortly be giving a sample of blood, or other fluid or solid tissue to confirm your diagnosis or to monitor your treatment. Although great progress has been made in the understanding of diseases and cancer in the last twenty years, we still have a lot to learn if we are to improve the cure rate further and learn how to prevent diseases including cancers occurring in the future. Many research projects require the use of cells taken from the cancer, blood, other body fluids, such as the fluid within the abdomen, and samples of tissues such as lymph glands in order to understand what has gone wrong. In addition we need many samples from healthy tissues. In order to help with such research, Newcastle is co-ordinating a local collection of stored tissue samples, blood and relevant fluids for use in research projects. This collection is called a "Biobank"

We would like to ask you if we can keep a small amount of the samples which we need to take as part of your investigation or treatment to use in research projects to help our understanding of diseases and cancer and help us to develop better ways of treating patients. The samples we keep come from surplus material left over after we have completed routine hospital tests and this will not involve any additional procedures.

The research we plan to do has been approved by the Newcastle & North Tyneside 1 Research Ethics Committee and will be regularly monitored by them. We occasionally send samples to other researchers in the UK and abroad. We only do this if the research they are undertaking is approved by their local ethics committee. Research may involve the testing of your DNA but your identity will not be revealed to the research team. Although research will not be conducted for the purposes of making money it is possible that some of the results will be of value to commercial companies, for example in the development of new tests or treatments.

This work will not directly benefit you now but may help patients with cancer in the future. You do not have to give permission for your samples to be stored. If you decline it will not affect your medical care in any way.

What will happen if I take part?
If you give approval for samples to be stored and used for research purposes, these will be taken from the material left over after routine tests have been performed. The samples would be taken at various stages of treatment, which may include diagnosis, end of treatment and at any other time of assessment. The research samples would only be taken when we are sure that there has been enough taken for the other tests to establish your diagnosis.

What will happen to my specimens?
The samples will be stored in the Newcastle Cancer Centre Biobank or at another research laboratory, approved for the purpose. These samples may be stored for many years before they are used. The samples may be used to extract DNA (the genetic material inside a cell) which will also be stored. The samples are stored and labelled with a code number, which means that the only link between the sample code and any medical information is held at the local hospital. Samples released

from the Newcastle Cancer Centre Biobank for approved research projects may be transferred to other research laboratories only if they have approval from the appropriate ethical committee.

Researchers may be given access to some information about your health and diagnosis but they will not be given any personal information that will identify you. Similarly, any results from each research project will not be traceable back to you by either a researcher or your doctor. All information on your samples will be kept at the Newcastle Cancer Centre Biobank in accordance with current national regulations (the Data Protection Act and Caldicott guidelines).

How do I know that my specimens will only be used for ethically approved medical research?
Approval has been given by the Newcastle & North Tyneside Research Ethics Committee to perform research in Newcastle University on samples collected in the Biobank. The nature of this research will be regularly monitored by the committee. As we do not have the resources to undertake all aspects of cancer research, other centres may be carrying out approved research projects different from those carried out at our centre. It may be necessary from time to time to send them samples from patients treated at our centre. Samples will only be released to other research centres if they can demonstrate that they have equivalent approval from an appropriate ethics committee. Any samples sent out of Newcastle would remain anonymous to the researcher and there is no way that the research teams can find out who the sample came from.

Will I be told the results of research tests on my samples?
No. Nor will the result be given to your hospital doctor or general practitioner. The overall results of the research projects will be published in the scientific literature. In the future, if the research showed that there was a test which might be useful to you then you would be able to discuss the test with your doctor.

What will happen to the information gathered about me?
No personal identifiable information about you will be given to the scientists doing the research tests and any research carried out on your tissue that is linked to your medical condition will be done in a strictly anonymous way. A link will be retained with your clinical information but this will be through a coding system and the research team will not be informed of your identity. Information from your medical notes relating to diagnosis, family history of cancer and response to treatment will be kept in the Newcastle upon Tyne Hospitals NHS Foundation Trust and stored in a strictly confidential manner according to the Data Protection Act.

What are the possible risks of taking part?
There are no extra risks involved in collecting the samples to be stored for research.

Are there any possible benefits?
The information learned from studies using the specimens obtained from you may help to improve treatment for future patients with cancer and possibly lead to the prevention of these diseases.

Do I have to take part?
No. Your participation in this study is entirely voluntary. Whether or not you decide to allow your specimens to be stored for research purposes will not affect you being given the best possible treatment for your disease. If at any time in the future you change your mind and do not wish the specimens to be used for research, then as soon as you have told your doctor, we will destroy any samples remaining in the biobank, usually by incineration. However, data already produced using

your samples will not be destroyed and it will not be possible to destroy samples which have been sent in anonymous form to research teams

What should I do if I have any concerns about taking part?
If you have any problems, concerns or other questions about this study, please feel free to speak to the medical or nursing staff who are involved in your care, or you can phone the doctor in charge of overseeing the storage of samples in the Biobank; Professor Andy Hall, Newcastle University (0191) 246 4411

NO CELLS, TISSUE OR OTHER BODILY MATERIAL WILL BE TAKEN OR STORED FOR RESEARCH WITHOUT YOUR AGREEMENT

SUPPLEMENTARY CONSENT FOR GIFTING OF CLINICAL SAMPLES

Title of Project: **Use of Diagnostic Samples and Samples Taken During Therapy to Support Research into Cancer – to be stored in the Newcastle Cancer Centre Biobank**

Name of Researcher: **Professor Andrew Hall, on behalf of the Newcastle Cancer Centre Biobank**

You will shortly be giving a sample of blood or other fluid or solid tissue to confirm your diagnosis or to monitor your treatment.

Once your diagnosis has been made the remaining samples are very valuable for research. We therefore ask for your consent to allow use of any remaining sample for these purposes, by making a gift of the material to a research Biobank. A leaflet is available containing more information about the use of these samples and describing our policy for the safe keeping of tissue gifted in this way.

Your diagnosis and treatment will not be adversely affected in any way by giving consent. Any research studies in which your samples are used will have been approved by the Newcastle & North Tyneside Research Ethics Committee or an equivalent organisation. This is to ensure that the research is justified and meets current ethical standards. Research will be conducted anonymously, which means that your samples will be identified only by a code. Your personal details will not be accessible to researchers. Your confidentiality will be respected at all times.

Some research may include testing your DNA. Researchers will not know your identity and your samples will only be identified by an anonymised code. Your DNA will not be used for any other purposes other than approved medical research.

We may use the samples in association with commercial research partners but human tissues are never sold (this is illegal). Our partnerships are organised on a not-for-profit basis, with any resulting benefits being used directly to improve patient care or to enable us to perform more research. Although the research will not be conducted for the purposes of making money it is possible that some of the results will be of value to commercial companies, for example in the development of new tests or treatments.

Most of the research conducted using the samples we obtain is conducted in the UK, but as the work we undertake is part of an international effort we sometimes send samples to centres outside the country- for example to mainland Europe or the USA. If you do not wish your samples to leave the country you can indicate this on the form we ask you to sign.

Use of your tissue or cells for research in this way will not influence your treatment directly. Withholding consent will not affect your diagnosis or treatment in any way. If you do not wish to give consent, we guarantee that any sample surplus to needs for diagnosis is disposed of appropriately, following national guidelines.

It may be necessary to gather information from your medical records that is relevant to the research being conducted, such as the type of treatment you are receiving. This will only be done by a member of your healthcare team, or by someone that has a confidentiality agreement with the NHS Trust. Your personal details and identity will never be passed on to researchers. It may also be necessary for regulatory officials to check your medical records and laboratory data, to ensure that research is being carried out properly and in line with regulatory guidelines.

The donor should complete the whole of this sheet himself/herself
(Please initial your response to question 1 and then proceed to questions a-j below)

Q1. I agree to the use of my sample(s) after diagnosis for research	***Please initial here***

*Please **TICK** box yes to agree and box no to decline*	Yes	No
a. Have you read and understood the patient information sheet? (Please take a copy home with you to keep)		
b. Have you had an opportunity to discuss the Biobank and ask any questions?		
c. Have you had satisfactory answers to your questions from Prof/Dr/Sr ……..?		
d. Do you understand that you are free to withdraw your consent from the Biobank at any time without having to give a reason and without affecting your future medical care?		
e. Do you agree to donate any samples left over after clinical requirements, for use in future approved research projects?		
f. Do you understand that you will not be told the results of any tests which may be carried out on your samples		
g. Do you give permission for your medical information to be stored?		
h. Do you give permission for samples to be sent to centres outside the UK?		
i. Do you understand that relevant sections of your medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to you taking part in this research? Do you give permission for these individuals to have access to your records?		
j. Do you give permission for your DNA to be tested for the purposes of research?		

Patient (& Hospital number)	Date	Signature
Person taking consent	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes
Biobank Manager Contact Details: Professor Andy Hall, Tel: (0191) 246 4411

Appendix B. Manuscript

IJC
International Journal of Cancer

High-resolution imaging for the detection and characterisation of circulating tumour cells from patients with oesophageal, hepatocellular, thyroid and ovarian cancers

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Interest has increased in the potential role of circulating tumour cells in cancer management. Most cell-based studies have been designed to determine the number of circulating tumour cells in a given volume of blood. Ability to understand the biology of the cancer cells would increase the clinical potential. The purpose of this study was to develop and validate a novel, widely applicable method for detection and characterisation of circulating tumour cells. Cells were imaged with an ImageStream^X imaging flow cytometer which allows detection of expression of multiple biomarkers on each cell and produces high-resolution images. Depletion of haematopoietic cells was by red cell lysis, leukocyte common antigen CD45 depletion and differential centrifugation. Expression of epithelial cell adhesion molecule, cytokeratins, tumour-type-specific biomarkers and CD45 was detected by immunofluorescence. Nuclei were identified with DAPI or DRAQ5 and brightfield images of cells were collected. The method is notable for the dearth of cell damage, recoveries greater than 50%, speed and absence of reliance on the expression of a single biomarker by the tumour cells. The high-quality images obtained ensure confidence in the specificity of the method. Validation of the methodology on samples from patients with oesophageal, hepatocellular, thyroid and ovarian cancers confirms its utility and specificity. Importantly, this adaptable method is applicable to all tumour types including those of nonepithelial origin. The ability to measure simultaneously the expression of multiple biomarkers will facilitate analysis of the cancer cell biology of individual circulating tumour cells.

Detection of circulating tumour cells (CTCs) was first reported more than a century ago.[1] Interest in the clinical role of CTCs has increased with the development of improved technologies for their detection. The initial focus of research on cell-based detection has been on the enumeration of CTCs. Studies have sought evidence that the burden of tumour cells in the circulation of patients with advanced cancer provides prognostic or predictive information. High numbers of CTCs in patients undergoing chemotherapy for metastatic breast, colorectal and prostate cancer are associated with poor patient prognosis.[2–4]

The introduction of novel agents that target-specific molecular aberrations within cancer cells has driven exploration for biomarkers with which to inform accurate stratification of patients. Currently, biomarker profiles of tumour cells are measured on material obtained by surgical resection or invasive biopsy. CTCs are a source of disseminated malignant cells from which information about biological properties, or pharmacodynamic responses to novel therapeutics, may be obtained noninvasively.

Effective enumeration and characterisation depend upon a reliable method for the evaluation of CTCs. Detection of small populations of CTCs within the large number of normal blood cells represents a significant technical challenge. A single CTC may be detected in 7.5 ml of blood,[5] a volume that may contain up to 75 million leukocytes and 50 billion erythrocytes. Enrichment of the sample is required before tumour cell identification and characterisation can occur. This enrichment may consist of positive selection of the

Key words: circulating tumour cells, oesophageal adenocarcinoma, hepatocellular carcinoma, thyroid carcinoma, ovarian cancer, ImageStream^X imaging flow cytometry
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What's new?
Circulating tumour cells (CTCs) are disseminated malignant cells from which biological and therapeutic information may be obtained non-invasively. Detection of small CTC populations within the large number of normal blood cells is a challenge. This study describes a novel method for the detection and high-resolution imaging of CTCs. Unlike most other studies, CTC detection is not reliant upon expression of a single biomarker. The method is applicable to all cancers; the authors present preliminary results from four tumour types. The high quality of the images allows biological characterisation of the tumour cells and increases the clinical potential of the approach.

CTCs, positive depletion of the normal blood cells or a combination of the two approaches. A perfect method would remove completely the red blood cells, white blood cells and platelets, produce no cellular debris and recover all the CTCs. The majority of clinical studies reported have relied upon positive selection of tumour cells that express a single biomarker.[6–13] The antigen chosen most commonly is epithelial cell adhesion molecule (EpCAM).

We sought to develop a method for identification, quantification and characterisation of CTCs that would not rely upon expression of a single antigen and would be applicable to multiple tumour types. Additional aims were to obtain high-resolution images of the cells, avoid cell damage, achieve high sensitivity and specificity and have the ability to analyse the expression of multiple biomarkers in each tumour cell. We report the development of our method, and its validation with whole blood from patients with oesophageal, hepatocellular, thyroid and ovarian cancers.

Materials and Methods

Tissue culture

SK-GT-4 oesophageal adenocarcinoma cells (DSMZ, Braunschweig, Germany) and OVCAR-3 ovarian cancer cells (ATCC) were grown in RPMI supplemented with 10% foetal calf serum (FCS). ML1 thyroid cells (DSMZ, Braunschweig, Germany) were grown in DMEM supplemented with 10% FCS (Life Technologies, Paisley, UK). Huh-7 hepatocellular carcinoma cells (ATCC) were grown in DMEM with F12 Ham's nutrient and 10% FCS. All other reagents were purchased from Sigma-Aldrich (Poole, UK) unless stated otherwise.

Cells were maintained in exponential growth at 37°C in a humidified atmosphere, supplied with 5% CO_2 and discarded after the 30th passage. Cells were confirmed to be mycoplasma free (MycoAlert mycoplasma detection kit; Lonza).

Immunofluorescence

Cells were fixed by incubation in 0.4% formaldehyde for 20 min, or Phosflow Lyse/Fix buffer (BD, Oxford, UK) and permeabilised by incubation in Perm/Wash buffer (BD, Oxford, UK) for 1 hr at room temperature. Cells were incubated with 1:20 antipan-cytokeratin (clone C-11) PE (Cayman Chemical) and appropriate tumour-specific intracellular antibodies for 30 min at room temperature: 1:50 antisurvivin Alexa Fluor® 647 (Cell Signaling); 1:50 antiMUC16 which is known as carcinoma antigen 125 (CA-125) (Abcam) Alexa Fluor® 594, conjugated with an APEX™ kit (Invitrogen) as per manufacturer's instructions; 1:50 antialpha faetoprotein Alexa Fluor® 594 (Cell Signaling). Membrane antibodies and nuclear stains were added and incubated for 1 hr at room temperature: 1:20 anti-CD45 (clone H130) V450 or PE:Cy7 (BD Biosciences); 1:20 anti-EpCAM CD326 (clone 9C4) Alexa Fluor® 488 (Biolegend); DAPI or DRAQ5 (Biostatus, UK). Cells from thyroid cancer patients were incubated first with 1:50 antisodium:iodide symporter (NIS; Milipore) followed by anti-mouse Texas Red (Life Technologies, UK). Subsequent incubations were as described above except that the anti EpCAM CD326 (clone 9C4) was conjugated to PerCP:Cy5.5 (Biolegend). Cells were washed in 500 µl of Perm/Wash buffer and recovered by centrifugation at 500 g for 5 min and either analysed immediately or stored as a pellet at 4°C until analysis.

High-resolution flow cytometry

Cells were resuspended in phosphate-buffered saline (PBS) and divided into 60 µl aliquots. Aliquots were analysed with an ImageStream^X (Amnis) image flow cytometer with an 8-µm core at 60 mm/sec with 7% Speed Beads®. Speed Beads® are a 1 µm polystyrene beads that allow calibration of the flow and focus of the ImageStream^X. Fluorochromes were excited with 405, 488, 561 and 642 nm lasers and light emitted by the fluorescently-labelled cells was collected through a ×40 objective. Of the twelve channels available, channels 1 and 9 were reserved for brightfield images. The other channels were set to collect magnified emitted light with two CCD cameras, each spatially resolved into five distinct spectral bandwidths, over a range of wavelengths between 430 and 745 nm. Single-colour reference samples for each fluorochrome were generated by inclusion of cells that had been incubated with each antibody separately. A compensation matrix was built with the data from single-colour reference samples to allow removal of spectral overlap to adjacent channels from each detection channel. The diameters of individual cells detected were measured and are given as means ± standard errors of the mean for different populations of cells.

Detection of cells in whole blood

Ethical approval for the study was obtained from the Newcastle and North Tyneside Research Ethics Committee. An initial predraw of 4 ml of blood was discarded to reduce

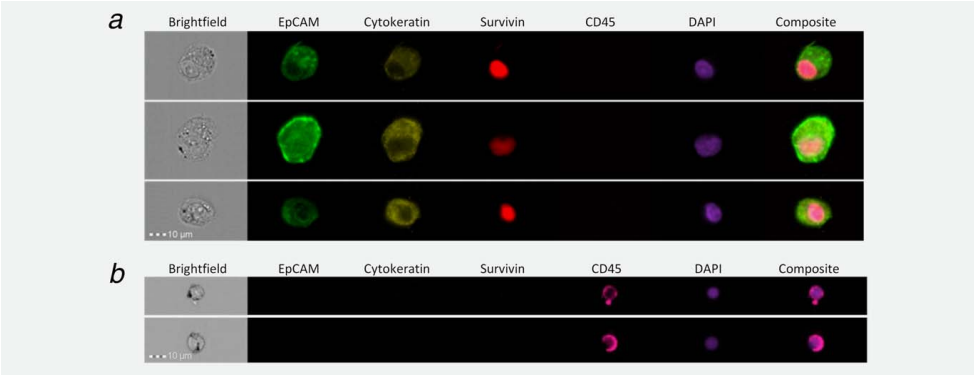

Figure 1. EpCAM, cytokeratin, survivin and CD45 expression in oesophageal adenocarcinoma cells and in white blood cells. SK-GT-4 cells were grown to 80% confluence in routine culture medium, trypsinised and 1×10^6 cells fixed with 1% formalin. Cells were permeabilised by incubation with 0.3% saponin, incubated overnight with antibodies against cytokeratins 4, 5, 6, 8, 10, 13 and 18, and CD45, and incubated subsequently with antibodies against EpCAM and survivin. Cells were washed, re-suspended in 100 µl and 2 µl DAPI added. Cells were visualised with an ImageStream^X flow cytometer with the lasers set to emit excitation at 405, 488, 561 and 658 nm (*a*). Red blood cells were removed from whole blood by ammonium chloride lysis, the remaining blood cells were concentrated by centrifugation, fixed, permeabilised and incubated with antibodies against EpCAM, cytokeratins 4, 5, 6, 8, 10, 13 and 18, survivin and CD45 as in (*a*). Blood cells were concentrated, incubated with DAPI and visualised (*b*). Images were collected with a ×40 objective with the wavelengths for the collection channels set at: 480–560 nm, EpCAM; 560–595 nm, cytokeratins; 745–800 nm, CD45; 430–505 nm, DAPI; and 642–745 nm, survivin.

contamination with epithelial skin cells. Blood samples were collected in Transfix collection tubes (Cytomark, UK) to store for up to 24 hr at 4°C or BD Vacutainer EDTA tubes (BD Biosciences) for immediate use.

For each cell recovery experiment, 12 ml-blood samples were collected from healthy volunteers. Cells from cancer cell lines were trypsinised and resuspended in media. Cells were counted with an improved Neubauer haemocytometer (Hawksley, UK) and were diluted twice by 1 in 10. The appropriate volume of cells containing 2,000, 200 or 20 cells was added to 4 ml blood samples to give 500, 50 and 5 malignant cells/ml of whole blood.

To determine the contribution of the final stage of the imaging flow cytometric analysis to the overall recovery rates, 500 unprocessed cells were analysed directly with the ImageStream^X flow cytometer. Cells were imaged in the brightfield channel and images with cellular morphology were counted.

Analysis of patient samples

Whole blood samples were obtained from patients undergoing treatment for oesophageal adenocarcinoma, hepatocellular carcinoma, thyroid carcinoma or ovarian cancer at the Newcastle-upon-Tyne and Gateshead NHS Foundation Trusts, UK. Patient clinical data was recorded for each patient in accordance with ethical approval. In addition whole blood samples were obtained from healthy volunteers who had no medical history of any current or previous cancer. CTCs were defined by the presence of at least one tumour-specific antigen, the absence of CD45, the presence of a nucleus and cellular morphology as assessed by brightfield imaging. CTC images were verified independently by two members of the research group.

Depletion of haematopoietic cells

Patient and healthy volunteer samples were processed to enrich for nonhaematopoietic cells prior to analysis. Cells were transferred into 50 ml Falcon tubes, incubated in 5 ml of 5% bovine serum albumin (BSA) in AutoMACS rinse solution (Miltentyi Biotec, Germany). Human FcR blocking reagent (Miltenyi Biotec, Germany) was added directly to the blood to a final dilution of 1:40 to prevent nonspecific antibody binding. Red blood cells were lysed, and all other cells fixed, by incubation in BD Phosflow Lyse/Fix buffer 1:20 (v:v; BD Biosciences) for 15 min at 37°C. Fixed, unlysed cells were collected by centrifugation at 500 g at room temperature for 8 min. The supernatant was discarded and the cells were resuspended in 500 µl RoboSep buffer (Stemcell Technologies, UK) in a polystyrene Falcon tube (BD Biosciences).

White blood cells were removed with an EasySep human CD45 depletion kit (Stemcell Technologies, UK) as per the manufacturer's instructions. Briefly, antibodies against CD45, bound in tetrameric complexes were added to the cell suspension and incubated for 15 min at room temperature. Dextran-coated magnetic nanoparticles were added and incubated with the cells for 10 min at room temperature. The cell suspension was diluted in 5 ml of Robosep buffer and placed in an EasySep^{TM} big easy magnet (Stemcell Technologies, UK) for 10 min at room temperature. The unretained cell

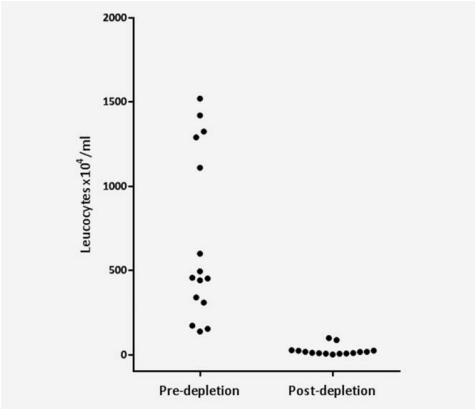

Figure 2. Enrichment for nonhaematopoietic cells. Cultured malignant cells were added to 4 ml of whole blood and cells were incubated with tetrameric antibody complexes against CD45 and dextran-coated magnetic particles for 1 hr and placed in an EasySep Big Easy magnet. Cells not attracted to the magnet were recovered. The number of leukocytes was counted with a haemocytometer prior to and after depletion. Erythrocytes were removed by lysis and platelets by centrifugation at 250 g.

fraction was decanted into a clean tube by inversion of the sample and magnet. The recovered cells were centrifuged at 250 g for 5 min, resuspended in 1 ml Perm/Wash buffer (BD, Oxford, UK), incubated for 1 hr at room temperature and processed for analysis by image flow cytometry.

Results

Image flow cytometry of malignant cells

Four distinct tumour types were selected for development of a universal method for detection of CTCs. Expression of EpCAM and cytokeratins 4, 5, 6, 8, 10, 13 and 18 was chosen for detection of the malignant cells. In addition, detection of expression of tumour- or tissue-specific markers was included: for hepatocellular carcinoma, alpha-faetoprotein; for thyroid carcinoma, thyroglobulin and sodium:iodide symporter (NIS) and for ovarian cancer, cancer antigen 125 (CA-125). There is no accepted tumour-specific marker for oesophageal adenocarcinoma, but survivin expression is reported to be high and measurement of its expression was included for this tumour type.

Initially, detection was optimised with established cell lines. SK-GT-4 oesophageal adenocarcinoma cells were incubated with fluorescently-conjugated antibodies against EpCAM, cytokeratins 4, 5, 6, 8, 10, 13 and 18, and with DAPI, a fluorescent dye that binds DNA. A fourth conjugated antibody against leukocyte common antigen CD45 was included because it would allow subsequent discrimination of leukocytes. Cells were analysed for expression of the antigens with an ImageStream^X image flow cytometer. Representative images from three cells are shown in Figure 1*a*. The different images obtained from a single cell are shown in each horizontal panel. The brightfield image of the cell shown on the left hand side of each panel allows visualisation of the nucleus, plasma membrane and overall cell morphology. All three cells have cytoplasmic immunoreaction for EpCAM, with some evidence of intracellular vesicular accumulation and membrane localisation. The immunoreaction for the cytokeratins was less intense, but evident in the cytoplasmic compartment of all cells. Survivin expression was detected in the nuclei of the cells coincident with the localisation of the DAPI DNA dye. The composite images shown on the right hand side of the panels confirm localisation of EpCAM and the cytokeratins in the cytoplasmic and membrane compartments of the cells and the distinct localisation of survivin in the nuclei. Expression of CD45 was not detected in SK-GT-4 oesophageal adenocarcinoma cells.

Hepatocellular Huh-7 cells, ML1 thyroid cells and OVCAR 3 ovarian cells were analysed as described above except that the antibody against survivin was replaced with antibodies against alpha-faetoprotein, thyroglobulin and NIS, and CA-125, respectively. Images of comparable quality to those shown in Figure 1*a* were obtained for all three cell lines (data not shown). Alpha-faetoprotein, thyroglobulin and NIS, and CA-125 were detected in Huh-7, ML1 and OVCAR 3 cells, respectively. These results demonstrate the applicability of the method to the detection of multiple tumour types, the measurement of tumour-type-specific biomarkers and the high quality of the images that may be obtained.

Detection of malignant cells in, and recovery from, whole blood

It was important to demonstrate the specificity of our method with whole blood from healthy individuals. Blood was collected, red blood cells were lysed and the remaining blood cells collected by centrifugation. These blood cells were incubated with antibodies against EpCAM, cytokeratins 4, 5, 6, 8, 10, 13 and 18, survivin and CD45, centrifuged at low g-force to remove platelets and analysed for expression of the antigens by image flow cytometry (Fig. 1*b*). The brightfield images demonstrate that the cells detected are of smaller diameter (12.2 $\pm$ 0.2 μm) than the SK-GT-4 oesophageal cells (20.3$\pm$ 0.2 μm; unpaired *t* test, $p < 0.001$); nearly all express CD45. Fifty-three blood samples from healthy individuals have been analysed with the tumour-specific antibodies. No cells were detected with morphology consistent with a malignant cell and expression of a tumour-specific antigen. These results indicate that the detection method will discriminate effectively nonhaematopoietic cells from haematopoietic cells.

Despite the ability of the ImageStream^X flow cytometer to image 2,000 cells/sec, the large number of cells in whole blood means that analysis is extremely lengthy and produces an enormous amount of data for analysis and storage. We investigated the best method with which to enrich blood for nonhaematopoietic cells. For these experiments, known numbers of

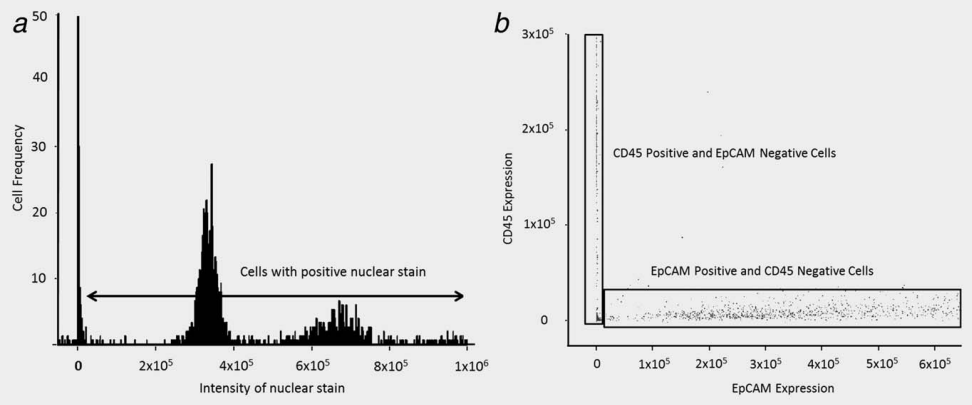

Figure 3. Discrimination of the malignant cell population in whole blood from the residual leukocytes after positive depletion of blood cells. Cultured malignant SK-GT-4 cells were added to 4 ml of whole blood. Erythrocytes were removed by ammonium chloride lysis and platelets by centrifugation at 250 g. The cells in the supernatant were incubated with tetrameric antibody complexes against CD45 and dextran-coated magnetic particles for 1 hr and placed in an EasySep Big Easy magnet. The cells not attracted to the magnet were recovered and analysed in an ImageStream^X flow cytometer as described in the legend to Figure 1. The number of images at each DAPI fluorescence intensity is shown (*a*). The intensity of fluorescence for the CD45 antibody is compared with the intensity of fluorescence of the EpCAM antibody for all cells distinguished (*b*).

cultured malignant cells were added to samples of whole blood and then the malignant cells were purified and analysed by image flow cytometry. Positive selection of the malignant cells involved identification of a universally expressed antigen or differential separation on the basis of density. All methods tested gave low recovery of the malignant cells and the cells that were recovered were damaged physically as assessed by the images produced (data not shown). We therefore developed a method for positive depletion of haematopoietic cells. After initial lysis of the erythrocytes; the leukocytes, platelets and malignant cells were incubated with tetrameric antibody complexes against CD45 and dextran-coated magnetic particles. The cells were placed in a magnet and those not attracted to the magnet were recovered. The method was optimised to minimise loss of nonhaematopoietic cells whilst maximising depletion of CD45 positive cells.

Depletion of leukocytes was consistently 95 ± 0.8% (Fig. 2). The recovered cells were incubated with detection antibodies and DAPI, centrifuged at 250 g to remove platelets and analysed by high-resolution image flow cytometry as described above. Analysis of cells enriched from 1 ml of blood takes 20 min in the ImageStream^X flow cytometer whereas analysis of 1 ml of blood without prior enrichment takes 180 min. Images of the malignant cells and residual leukocytes were similar to those shown in Figure 1, which confirms that minimal damage to the malignant cells is caused by this enrichment method of positive blood cell depletion (data not shown).

The malignant cells are distinguished from residual leukocytes by expression of epithelial cell- and tumour-specific antigens, absence of expression of CD45, and by their morphology and larger size. Analysis of the images obtained with the IDEAS Software enables automatic discrimination of the two cell populations. The first selection is based upon the intensity of the nuclear dye retained by the cells (Fig. 3*a*). The sharp peak around zero contains beads and small particles of debris. The peak of intensity between 3 and 4 × 10^5 fluorescence units contains single leukocytes. The third peak of intensity between 5 and 8 × 10^5 fluorescence units contains malignant cells and doublets of white blood cells and is analysed further. Subsequent selection is based upon absence of CD45 expression and presence of expression of EpCAM, cytokeratins and survivin, alpha-faetoprotein, thyroglobulin, NIS or CA-125. The effectiveness of the discrimination is illustrated in Figure 3*b*; one population of cells expresses CD45 but not EpCAM while the second population expresses EpCAM but not CD45. Cells that express one or more of the epithelial- or tumour-specific antigens and do not express CD45 are selected automatically with the IDEAS Software. The images of each of these cells are examined visually to confirm that they have a cellular morphology as shown in Figure 1 and that the IDEAS Software is able to distinguish the malignant cells from any residual haematopoietic cells.

We processed, imaged and analysed 5 ml blood samples from 24 healthy volunteers as described above. Blood was enriched by CD45-positive cell depletion, incubated with the detection antibodies and analysed by ImageStream^X flow cytometry. The majority of the residual white blood cells were detected with the CD45 antibody. None of the cells imaged within the healthy volunteer samples met the criteria for classification as a CTC.

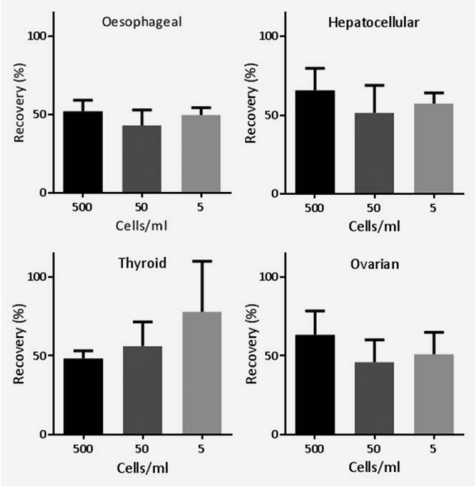

Figure 4. Retrieval of malignant cells from whole blood. SK-GT-4, Huh-7, ML1 and OVCAR 3 cells were added to 4 ml of whole blood to give a final concentration of 500, 50 and 5 malignant cells/ml. The samples were enriched for malignant cells by depletion of blood cells and the residual cells were incubated with fluorescent antibodies and nuclear dye and analysed by high-resolution flow cytometry as described in the Materials and Method. The mean recoveries (±SEM) across the four tumour types were 57.3 ± 3.6, 49.2 ± 3.9 and 59.0 ± 5.6% from 500, 50 and 5 cells/ml of blood, respectively. Experiments were replicated at least thrice.

The efficiency of recovery of malignant oesophageal, hepatocellular, thyroid and ovarian cells from whole blood was evaluated. The cell recovery across the four tumour types was 57.3 ± 3.6, 49.2 ± 3.9 and 59.0 ± 5.6% from 500, 50 and 5 cells/ml of blood, respectively (Fig. 4). We investigated specifically the recovery during the final analysis with the image flow cytometer. The recovery during analysis of known numbers of cells by image flow cytometry was 89.2+/−6.2%, which means that the recovery during the red blood cell lysis, white blood cell depletion, centrifugation and antibody labelling steps was 61.9% to give an overall recovery of 55.2%.

Detection and analysis of circulating tumour cells

To validate the method, blood samples from six individual patients with oesophageal, hepatocellular, thyroid and ovarian cancer were analysed essentially as described above. Representative images of tumour cells detected for each of the four tumour types are shown in Figure 5. The morphology of the oesophageal tumour cells detected was similar to that of the cultured cells. All of the oesophageal CTCs detected expressed EpCAM, cytokeratins and survivin. CTCs were detected in two of the six oesophageal adenocarcinoma patients analysed (Table 1). None of these patients had macroscopic evidence of metastatic disease. The mean diameter of all the circulating oesophageal adenocarcinoma cells detected was 17.2 ± 0.4 μm.

CTCs were detected in blood from four out of six patients with hepatocellular carcinoma (Figs. 5*b* and 5*c*). Some of the CTCs from patient seven expressed EpCAM but cytokeratins were not detected, and other cells expressed cytokeratins but EpCAM expression was not detected. None of the CTCs from this patient expressed alpha-faetoprotein. Alpha-faetoprotein was detected in cells from patient ten that did not express EpCAM or the cytokeratins (Fig. 5*c*). The morphology of the hepatocellular tumour cells varied. The diameter of the hepatocellular carcinoma cells was 21 ± 0.6 μm which is significantly larger than the diameter of the CTCs from all three other tumour types (unpaired *t* test, $p < 0.001$)

CTCs were detected in three of the six patients with thyroid cancer. The majority of these tumour cells expressed cytokeratins, thyroglobulin and NIS. EpCAM expression was low or undetectable. The highest number of CTCs was detected in blood from a patient with known metastatic disease. A third of their CTCs had clear membrane and cytoplasmic immunoreactivity for thryoglobulin, NIS and cytokeratins, no obvious morphological damage and well-defined oval nuclei (Fig. 5*d*). These CTCs stained intensely with DAPI possibly because they were aneuploid or were in the G2 stage of the cell cycle. The other cells expressed lower levels of cytokeratins, did not express detectable levels of thyroglobulin, NIS or EpCAM and stained less intensely with DAPI (Fig. 5*d*). These differences may represent heterogeneity of expression of biomarkers within the cells or the second group of cells may be undergoing cell death. The diameter of the circulating thyroid cancer cells was 16 ± 0.3 μm.

CTCs were detected in blood from four out of six patients with ovarian cancer. The cells all expressed EpCAM and cytokeratins. CA-125 expression was detected in around half of the tumour cells (Fig. 5*e*). The diameter of the CTCs detected in blood from ovarian cancer patients was 13.6 ± 0.59 μm. This diameter was significantly smaller than the diameters of CTCs detected in oesophageal adenocarcinoma, thyroid cancer and hepatocellular carcinoma patients ($p < 0.001$).

Discussion

We report a method for the detection and accurate characterisation of CTCs by high-resolution image flow cytometry. We demonstrate that this method is reproducible in samples from four tumour types. EpCAM was included within our panel of antigens, but could be replaced with other biomarkers for detection of nonepithelial malignant cells. Similarly as novel biomarkers are discovered, analysis of these could be incorporated. The method could be adapted also for measurement of pharmacodynamic biomarkers. The process of enrichment that we describe is based exclusively upon the positive depletion of haematological cells. Following this

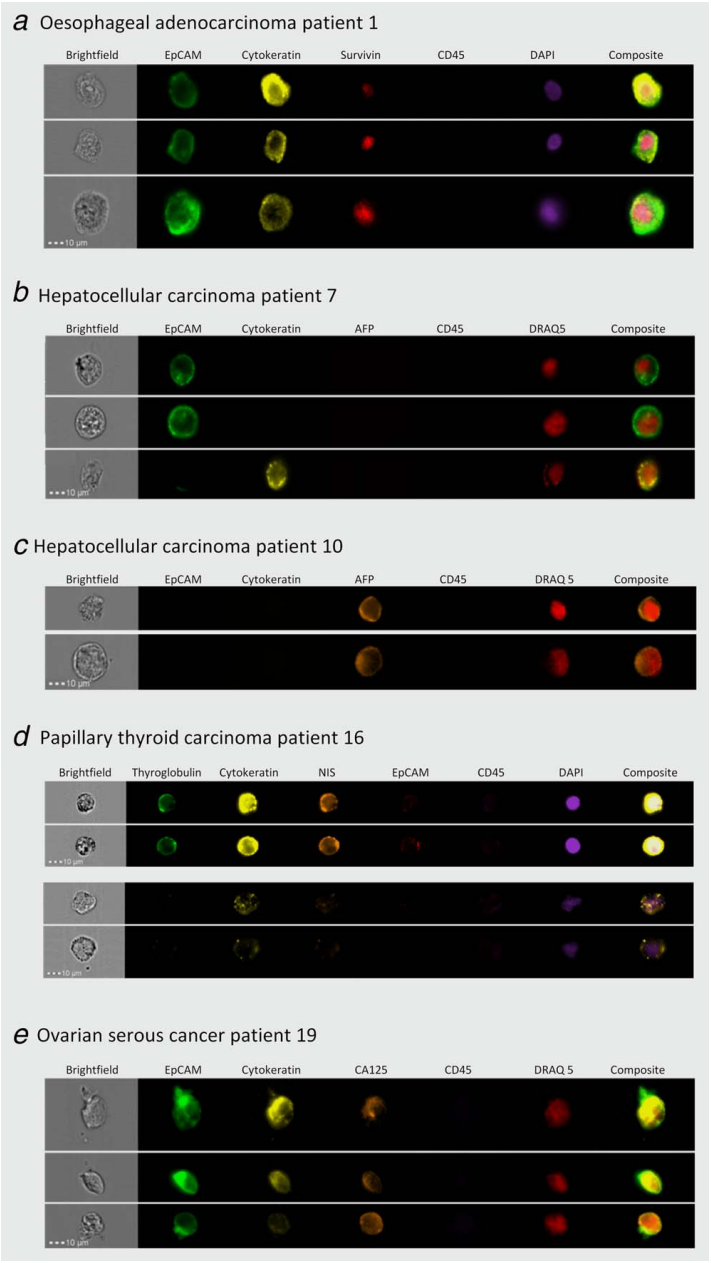

Figure 5. Detection of circulating tumour cells in blood from patients with oesophageal, hepatocellular, thyroid and ovarian cancers. Blood was obtained from patients, enriched for nonhaematopoietic cells and incubated with antibodies against EpCAM, cytokeratins 4, 5, 6, 8, 10, 13 and 18, CD45 and either survivin (oesophageal; *a*), alpha-faetoprotein (AFP) (hepatocellular; *b* and *c*), thyroglobulin and sodium:iodide symporter (thyroid; *d*) or CA-125 (ovarian; *e*). Cells were incubated with DAPI (*a* and *d*) or DRAQ5 (*b*, *c* and *e*) and analysed by high-resolution flow cytometry. Representative images of cells are shown. Small particles visible in some brightfield images represent the Speed Beads® that are added to allow calibration of the objective and camera.

Table 1. Patient demographics and numbers of circulating tumour cells in blood from patients with oesophageal, hepatocellular, thyroid and ovarian cancers

Patient no.	Tumour origin	Tumour type	Sex	Age	Disease stage[1]	Distant metastatic disease	Previous therapy	CTCs detected[2]	CTCs in 7.5 ml
1	Oesophagus	Adenocarcinoma	Male	59	T2N2M0	No	No	43	64
2	Oesophagus	Adenocarcinoma	Male	63	T3N2M0	No	No	17	27
3	Oesophagus	Adenocarcinoma	Male	83	T1N0M0	No	No	0	0
4	Oesophagus	Adenocarcinoma	Female	74	T3N1M0	No	No	0	0
5	Oesophagus	Adenocarcinoma	Male	65	T2N0M0	No	No	0	0
6	Oesophagus	Adenocarcinoma	Male	72	T3N2M0	No	No	0	0
7	Liver	Hepatocellular carcinoma	Male	70	T1N0M0	No	TACE[3]/SIRT[4]	20	37
8	Liver	Hepatocellular carcinoma	Female	62	T2N0M0	No	TACE[3]	0	0
9	Liver	Hepatocellular carcinoma	Male	93	Unknown	No	No	0	0
10	Liver	Hepatocellular carcinoma	Male	75	T3bN0M0	No	No	9	16
11	Liver	Hepatocellular carcinoma	Male	81	T1N0M0	No	TACE[3]	2	4
12	Liver	Hepatocellular carcinoma	Female	85	T1N0M0	No	TACE[3]	2	4
13	Thyroid	Medullary thyroid cancer	Male	49	T2N2M1	Yes	Surgery	4	4
14	Thyroid	Multifocal follicular variant of papillary thyroid cancer	Female	29	T1aN0M0	No	Surgery Thyroxine	0	0
15	Thyroid	Follicular thyroid cancer	Female	47	T2N0M0	No	Surgery Thyroxine Radioiodine	1	1
16	Thyroid	Papillary thyroid cancer	Male	61	T4N2M1	Yes	Surgery Thyroxine Radioiodine radiotherapy	118	118
17	Thyroid	Follicular variant of papillary thyroid cancer	Female	52	T3N0M0	No	Surgery Thyroxine Radioiodine	0	0
18	Thyroid	Follicular variant of papillary thyroid cancer	Female	50	T2N0M0	No	Surgery Thyroxine Radioiodine	1	1
19	Ovary	Serous	Female	50	FIGO Stage 4	Yes	No	30	45
20	Ovary	Serous	Female	66	FIGO Stage 3c	Yes	No	15	23
21	Ovary	Mucinous	Female	75	FIGO Stage 3c	Yes	No	5	8
22	Ovary	Serous	Female	54	FIGO Stage 3c	Yes	No	0	0
23	Ovary	Serous	Female	85	FIGO Stage 4	Yes	No	4	6
24	Ovary	Serous	Female	72	FIGO Stage 3c	Yes	No	0	0

[1]Disease stage is provided according to the latest UICC TNM classification for oesophageal, hepatocellular and thyroid cancer, and the FIGO classification is shown for ovarian cancer.
[2]The volume of blood analysed was 5 ml for patients with oesophageal and ovarian cancers, 4 ml for those with hepatocellular carcinoma and 7.5 ml for thyroid cancer patients.
[3]Transarterial chemoembolisation.
[4]Selective internal radiotherapy treatment.

depletion, CTCs are distinguished from residual leukocytes and cellular debris by analysis of the expression of multiple antigens and by examination of cellular morphology in the high quality images.

The main focus of CTC research has been the value of CTC enumeration for prognosis discrimination in patients with metastatic disease and for prediction of response to cytotoxic therapy. Levels of CTCs are associated with overall

survival in pre- and on-treatment patients with metastatic breast cancer, metastatic colorectal cancer and castration-resistant prostate cancer.[2,3,14–19] The numbers of CTCs detected in patients with metastatic cancer are often low, and because detection of a single CTC may determine whether a patient is categorised into a good or a bad prognostic group,[2,3] it is important that all CTCs are detected, not only specific subpopulations. A strength of our method is that it permits detection of heterogeneity within a patient's CTC population (Fig. 5*d*).

There is considerable interest in the analysis of CTCs as a means of studying the biology and behaviour of metastatic cancer. Metastatic disease is frequently difficult to biopsy and treatment is based usually on analysis of the primary tumour. Specific protein expression in metastatic disease may differ from that of the primary tumour.[20,21] The capability to detect multiple antigens simultaneously enables detailed molecular characterisation of CTCs and may provide an accurate assessment of the biology of the underlying metastatic disease.

It is important that the specificity of CTC detection is high. There is no consensus definition as to what constitutes a CTC partly because of the large number of techniques used for their detection. Variation in the phenotypic criteria by which CTCs are defined results in different CTC counts with varying degrees of clinical significance.[22] The majority of studies define CTCs based upon positive and negative antigen expression. In some studies, molecular definition is combined with cell morphology. The high-resolution imaging that we describe allows us to discriminate objects that might otherwise have been included in the enumeration of CTCs. Some studies[11,23–32] have reported substantially larger numbers of CTCs than have been identified in the present study or in other reports.[2,3] It is possible that such high numbers reflect inclusion of noncellular objects and cellular debris.

Analysis of CTCs without enrichment is achievable with an ImageStream^X flow cytometer, but the time required to process an unenriched sample precludes realistic routine clinical application. Analysis of one unenriched 5 ml blood sample would take 15 hr compared to 1 hr 40 min for an enriched sample. The losses in our method occur predominantly during the enrichment and antigen detection stages. The recovery of cells during the final analysis with the ImageStream^X flow cytometer is 89.2%. The overall loss from the procedure is 44.8%, of which 38.1% is lost during the enrichment and 6.7% during the image collection. The EasySep CD45 depletion kit achieved the optimal recovery of CTCs without generating large quantities of cellular debris. Recovery rates are comparable with other methods in which leukocytes are depleted positively[33,34] and were consistent over a range of cell concentrations for all tumour types. Leukocyte depletion in head and neck cancers has been evaluated by fluorescent activated cell sorting with a variety of commercially available anti-CD45 antibodies and magnetic particles including EasySep.[34] Recovery rates of up to 86% were reported but in these analyses, cells were added to buffy coat rather than whole blood, which should give higher recovery rates because the losses associated with red cell lysis are not considered.[35]

The most widely used method for cell-based CTC analysis is the CellSearch system (Veridex) which has FDA approval for use in metastatic breast, colorectal and prostate cancer. Enrichment of samples depends upon positive selection of CTCs that express EpCAM, which means that detection of malignant cells is limited to those of epithelial origin that express EpCAM. There is considerable heterogeneity in EpCAM expression in established epithelial cancer cell lines.[36] Epithelial tumour cells that undergo epithelial mesenchymal transition (EMT) lose EpCAM expression and EpCAM expression changes during the cell cycle.[37]

A single study has attempted comparison of the ImageStream^X and CellSearch[38] by analysis of PANC-1 pancreatic cancer cells and reported that the accuracy of enumeration was lower with the ImageStream^X. The authors enriched the PANC-1 cells with different methods prior to analysis with the two platforms which, because the method of enrichment affects recovery rates, means that the two detection rates are difficult to evaluate.

There are few reports in the literature about the analysis of CTCs in oesophageal adenocarcinoma. Survivin mRNA was detected by RT-PCR in peripheral blood of patients with a variety of gastrointestinal tumours including oesophageal adenocarcinoma.[39] A recent study using the CellSearch platform assessed CTC numbers in patients with advanced oesophagogastric adenocarcinoma undergoing palliative chemotherapy.[40] The study was ended prematurely due to the loss of commercial funding. In 11 patients with advanced oesophageal or oesophagogastric junctional tumours, four were found to have CTCs. In thyroid cancer, there is again a lack of evidence for the value of CTC detection. A single study evaluated the detection carcinoma embryonic antigen (CEA) mRNA by RT-PCR in 121 patients undergoing surgery for thyroid cancer[41] and detected CTCs in 5% of patients.

EpCAM-positive CTCs were detected with the CellSearch system in 18 of 59 patients with hepatocellular carcinoma[42] and an association between presence of CTCs and overall survival reported. In another study, CTCs were found in 28% of HCC patient samples analysed with the CellSearch system but in 100% of samples analysed with an EpCAM-independent filtration method.[43] In a third study, multi-immunofluorescence identified considerable heterogeneity within CTC populations in HCC patients[44] and changes in the ratio of epithelial to mesenchymal cells were associated with a longer time to disease progression.

There is more extensive literature about the role of CTCs in ovarian cancer patients. Several studies have isolated a mononuclear cell fraction by density gradient separation followed by positive selection of CTCs based on their expression of epithelial antigens, usually a single antigen.[45,46] In one study in which cells were enriched sequentially by epithelial

and leukocyte-specific antigen expression, up to 149 CTCs were detected per millilitre of blood in 61% of patients under evaluation for ovarian cancer.[47] A wide variation in the detection rates of CTCs in patients with ovarian cancer of between 12 and 100% have been reported.[5,46,48,49] The highest number of CTCs reported is 3,118/ml of blood.[45]

A large number of methods with different strengths are described for the enumeration and characterisation of CTCs. It is unlikely that a single technique will be suitable for all research and clinical applications. The principle strengths of the method we describe are the quality of the images produced, the lack of positive selection of CTCs, its applicability to all tumour types, and the ability to characterise the biology of the cancer cells.

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