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Optimization of a size-based enrichment method for improved circulating tumour cell detection in metastatic breast cancer patients

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Abstract

Circulating tumour cells (CTCs) are tumour cells that have separated from the primary tumour or metastasis and entered the bloodstream. CTCs have shown to be promising diagnostic and prognostic biomarkers for several different cancers. Numerous approaches for detecting CTCs in the peripheral blood are available, including both marker-dependent and marker-independent techniques. This study aimed to optimize and evaluate a marker-independent, size-based method for the enrichment of CTCs from patients with metastatic breast cancer.

The breast cancer cell lines MCF-7 and ZR-75-1 spiked into blood from healthy volunteers were used as a model system for the validation of the VYCAP size-based filtration technology. The cells were stained with immunofluorescently labelled antibodies, and microscopy was used to validate the results of the staining and to enumerate the cells. Optimization of the method was done by testing different blood collection tubes, as well as fixation and permeabilization reagents. The TransFix blood collection tube, in combination with the FIX&PERM cell fixation and permeabilization kit, were determined to be optimal for this procedure.

CTCs were isolated from patients with metastatic breast cancer using the VYCAP size-based filtration system. Analysis of 7 patient blood samples detected CTCs and CTC clusters in only 1/7 (14%) of the samples.

In conclusion, optimal conditions for cell line cells were established, and cells were isolated with high recovery rates. Still, clinical validation of the size-based enrichment technique requires further investigation. More patient samples are necessary to assess clinical relevance.

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Abbreviations

Abbreviation	Explanation
CTC	Circulating Tumour Cell
HRT	Hormone Replacement Therapy
DCIS	Ductal Carcinoma in Situ
HER2	Human Epidermal Growth Factor 2
TNBC	Triple Negative Breast Cancer
HR	Hormone Receptor
ER	Oestrogen Receptor
PR	Progesterone Receptor
EpCAM	Epithelial Cell Adhesion Molecule
EMT	Epithelial-to-Mesenchymal Transition
MET	Mesenchymal-to-Epithelial Transition
BCT	Blood Collection Tube
PBS	Phosphate Buffered Saline
BSA	Bovine Serum Albumin
DAPI	4’,6-diamidino-2-phenylindole
IF	Immunofluorescence
PBMC	Peripheral Blood Mononuclear Cell
PFA	Paraformaldehyde

1. Introduction

1.1 Breast Cancer

Breast cancer is a form of cancer that impacts the lives of an incredible number of women every year, being the most commonly occurring cancer in women, and one of the main causes of female deaths worldwide [1]. This cancer type arises in the epithelium of the ducts or lobules of the breasts, and over time the cancer may progress and eventually invade other tissues and distant organs[2]. Improving breast cancer therapy and prevention remains a huge area of research.

1.1.1 Epidemiology

According to the Word Health Organization, there were around 2.3 million new cases of breast cancer occurrences in the world in 2020, making it the most frequently occurring cancer of this year. There were around 685 000 deaths registered as a result of breast cancer this same year [3]. The American Cancer Society estimates the numbers of new cancer cases and related deaths in the United States each year, and predicts about 297.790 new cases of invasive breast cancer, and about 43.700 deaths from breast cancer in the United States in 2023 alone [4]. In Norway, breast cancer is by far the most frequent form of cancer in women, as it constitutes for 22.3% of all cancer cases in women [5]. There were 4023 new cases of breast cancer registered in Norway in 2021 (3991 women and 32 men), and 601 individuals lost their lives to the disease in Norway the same year [6].

There is a long list of risk factors linked to breast cancer, the two main factors being linked to age and gender. Even though breast cancer may occur in men, it primarily occurs in women. About 1 in 8 of women in the United States develop invasive breast cancer in the course of their life, whereas a man's lifetime risk of developing the disease is about 1 in 833 [7]. In addition, breast cancer primarily affects women over the age of 50 years old, with an 8.9% cumulative risk of developing breast cancer up until the age of 75 years. In the period 2014-2019, only about 4.3% of all new cases occurred in women under the age of 40 [5].

Compared to other types of cancer, there is a high hereditary risk related to breast cancer. About 5-10% of breast cancer cases can be linked to gene mutations, the most frequent being *BRCA1* or *BRCA2* gene mutations, but also including mutations in *PTEN*, *STK11*, *CDH1* and *PALB2* [1], [5]. Researchers believe that around 70% of women with a mutated *BRCA1* of *BRC2* gene will develop cancer by the age of 80 [9].

In addition to inherited risk factors, acquired characteristics like obesity, excessive alcohol use, and lack of physical activity also increase the risk of developing breast cancer [6]. Additionally, there is a risk associated with the prolonged exposure to hormones from hormone replacement therapy (HRT) used to reduce menopausal symptoms [10]. The age of first pregnancy, how many children one has, and whether or not one breast feeds are also factors that has shown to be associated with breast cancer risks [6].

1.1.2 Diagnostics and detection

Breast cancer originates either in the epithelium of the milk ducts (85%) or the lobules (15%) in the glandular tissue of the breast. At this early stage, the cancer causes little to no symptoms, whereas over time, the cancer may progress and eventually invade surrounding tissues like the lymph nodes, and eventually to other organs in the body. Spread of the cancer to distant tissues is known as metastatic breast cancer [2]. The first symptom of breast cancer is usually the sensation of a lump or area of thickened tissue in the breast, normally first discovered by the individual themselves [11]. A triple diagnostics method is applied once a patient is suspected to have breast cancer, including a clinical examination, image diagnostics (mammography) and biopsy [5].

One of the most important factors when it comes to the prognosis of an individual with breast cancer is early detection, which is obtained through early diagnosis and screening [12]. The ideal is to diagnose patients in the earliest stage possible and through this reduce the number of patients diagnosed in late stages of the disease where treatment may be less effective. Screening tests the healthy population to identify people who may have the disease, but not yet show any symptoms. Multiple studies show that a systematic screening program clearly reduces breast cancer mortality, and for this reason, all women in Norway between the ages of 50-69 years of age are offered a mammography every two years [5].

1.1.2.1 Subtypes and stages of breast cancer

Once a patient has got the breast cancer diagnosis, an important part of the diagnostics is to identify the subtype of their specific breast cancer. There are several different types of breast cancer, defined by where the cancer starts, its extent and biology [13]. The specific type of cancer also determines which treatment should be applied.

The common approach to cancer staging is the Tumour-Node-Metastasis (TNM) system which gives a tumour prognosis based on the extent of the primary **T**umour,

absence or presence and extent of regional lymph **N**ode metastasis, and the absence or presence of distant **M**etastasis [14]. Determining the type of cancer in combination with its TNM-stage is important to give the patient the proper cancer treatment. When using the TNM system, “Letters “T”, “N”, and “M” are used in combination with numbers to determine the size and location of the tumour, the spread to lymph nodes, and the degree of metastasis, respectively [15]. An overview of TNM-stages can be found in table 1 below.

Table 1: *The TNM staging system for breast cancer. The TNM system gives a tumour prognosis based on the extent of primary tumour “T”, extent of regional lymph node metastasis “N”, and the presence of metastasis “M”. Prognosis is given by using the letters T, N and M in combination with numbers to determine extent of disease.*

Class	Stage	Subgroup	Characteristics
Tumour "T"	TX		Not evaluated
	T0		No evidence of cancer
	Tis		Carcinoma in situ. No spread to surrounding tissues.
	T1	- T1mi - T1a - T1b - T1c	- ≤ 1 mm - > 1 mm ≤ 5 mm - > 5 mm ≤ 10 mm - > 10 mm ≤ 20 mm
	T2		> 20 mm ≤ 50 mm
	T3		> 50 mm
	T4	- T4a - T4b - T4c - T4d	- Growth into chest wall - Growth into skin - Growth into chest wall and skin - Inflammatory breast cancer
Lymph nodes "N"	NX		Not evaluated
	N0		No cancer found, or areas of cancer < 0.2 mm
	N1	- N1 - N1mi	- Spread to 1-3 axillary lymph nodes and/or internal mammary lymph nodes - Cancer in lymph node is > 0.2 mm ≤ 2 mm.
	N2		Spread to 4-9 axillary lymph nodes, or spread to internal mammary lymph nodes and not axillary lymph
	N3		Spread to 10 or more axillary lymph nodes, or spread to lymph nodes under clavicle or collarbone
Metastasis "M"	MX		Not evaluated
	M0		No evidence of distant metastasis
	M1		Evidence of distant metastasis

For many cancers, the TNM combinations are also often grouped into five less detailed stages numbered 0-IV. Stage 0 cancer is the earliest detectable stage. It is non-invasive and benign, and the most common form is called ductal carcinoma in situ (DCIS), referring to a highly treatable disease that is still restricted to the location of the originating breast epithelium [16]. Stage I and II cancers are still described as early-stage cancers. Stage I refers to the early stage of invasive breast cancer, where area of spread is still relatively small. Stage II cancers are also restricted to a limited region of the breast, yet larger than stage I. Stage III cancers have spread even further into the breast, and stage IV cancers are the most advanced cancers and includes spread to distant organs [17]. The stages are divided into subgroups, and an overview can be found in table 2.

Table 2: *Breast cancer stages 0 – IV and their corresponding TNM classification. Breast cancer stages are divided into five groups numbered 0 – IV, and each stage corresponds to a certain TNM class. This table shows which breast cancer stage corresponds to which TNM stage, in addition to the extent of the certain stage.*

Overall stage		TNM category	Extent of disease
0		Tis, N0, M0	Non-invasive, localized within breast
I	IA	T1, N0, M0	- Locally invasive - Tumour is < 2cm
	IB	T0, N1mi, M0 T1, N1mi, M0	- Locally invasive - Tumoir is < 2 cm
II	IIA	T0, N1, M0 T1, N1, M0 T2, N0, M0	- Locally invasive - No tumour or tumour < 12cm, with tumour cells in 1-3 lymph nodes
	IIB	T2, N1, M0 T3, N0, M0	- Locally invasive - Tumour is > 2cm but < 5 cm, with small areas of cancer cell in lymph nodes
III	IIIA	T0, N2, M0 T1, N2, M0 T2, N2, M0 T3, N2, M0 T3, N1, M0	- Locally advanced - No tumour or tumour > 5 cm, with tumour cells in 4-9 lymph nodes
	IIIB	T4, N0, M0 T4, N1, M0 T4, N2, M0	- Locally advanced - Spread to skin of breast or chets wall
	IIIC	T(any stage), N3, M0	- Locally advanced - No tumour or tumour of any size, with spread to lymph nodes and skin
IV		T(any stage), N(any stage), M1	- Metastatic - Tumour of any size, with or without spread to lymph nodes

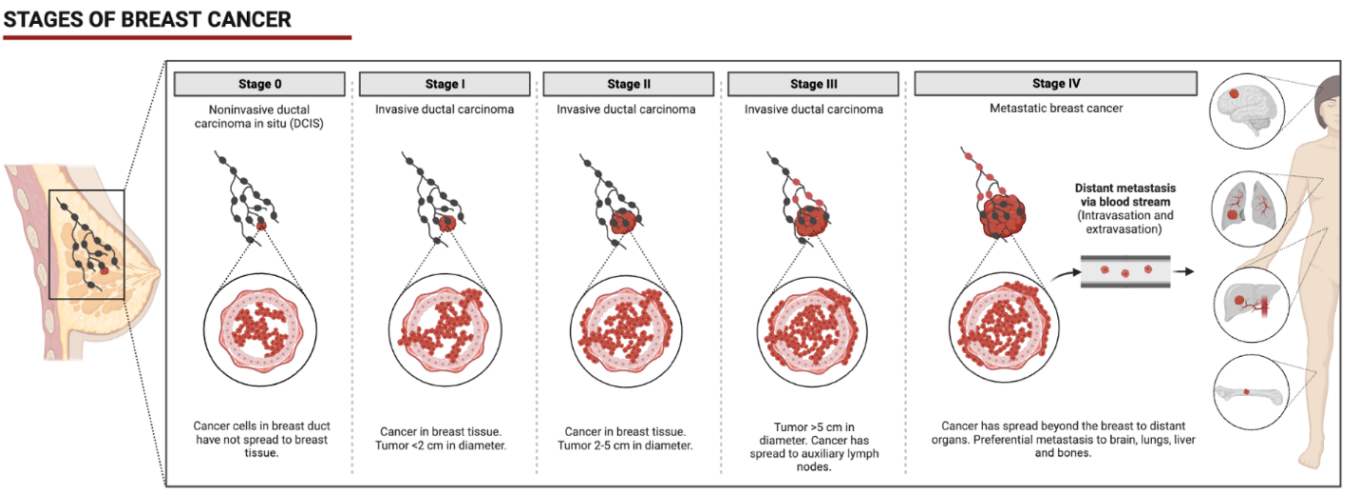

Figure 1: Breast cancer stages 0-IV*. Stage 0 diseases are non invasive. Stage I tumours are less than 2 cm, and stage II tumours are between 2-5 cm. A tumour of stage III is larger than 5 cm, and stage IV cancers represent cancers that have spread to distant organs. Reprinted from "Stages of breast cancer" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates/figures.*

An additional way of classifying tumours is by dividing them into molecular subtypes based on gene expression profiling. Breast cancer tumours can be classified into five molecular intrinsic subtypes, often based on the 50-gene signature known as PAM50. The subtypes are known as Luminal A, Luminal B, Normal-like, Human epidermal growth factor 2 (HER2) enriched, and triple negative breast cancer (TNBC) [18]. The scientific name for HER2 is ERBB2, however HER2 is normally used clinically. Additionally, breast cancer cells are classified as being either hormone receptor positive (HR+) or hormone receptor negative (HR-). Most breast cancer cells express hormone receptors that pick up oestrogen and progesterone signals that promote cell growth and accordingly, many breast tumours are positive for immunooestrogen receptors (ER+), progesterone receptors (PR+) or both (ER/PR+) [19]. Luminal A cancers are characterized as ER/PR+, and HER2-. These cancers usually have a low level of Ki-67, which is a protein associated with high cell proliferation rate, and the cancer usually has a good prognosis. Luminal B cancers are also HR+ but has a high level of Ki-67. Normal-like breast cancer is also HR+, but with a slightly worse prognosis than Luminal A cancers. HER2-enriched breast cancer is HR- and HER2+. Lastly, TNBC is the most aggressive form of breast cancer and is associated with the BRCA1 mutation [20].

1.1.3 Treatment

The treatment of breast cancer depends on the specific type of cancer and its corresponding stage. There are several treatment methods available, including local treatments like surgery and radiation, or systemic treatments like chemotherapy, hormone therapy, targeted drug therapy and immunotherapy [21]. It is normal for most patients to have some type of surgery as part of their treatment, which implies either a total mastectomy, or a breast-conserving surgery [22, 23]. Surgery is often done in combination with other treatment methods. Stages I-II cancers are normally treated with a breast-conserving surgery and subsequent radiation therapy, whereas a more aggressive stage III cancer usually requires neoadjuvant chemotherapy (before surgery) to down-size the tumour before a mastectomy can be performed. Adjuvant chemotherapy after surgery is also often given with the goal of killing cancer cells that might have been left behind during surgery and can not be picked up during imaging[21]. Radiation therapy is also used in combination with surgery if the cancer has spread to many lymph nodes, or if the cancer has spread to other parts of the body [21]. For patients that receive breast-conserving surgery, whole-breast radiation is performed to reduce the local recurrence rate.

For patients with stage IV breast cancer, prognosis is poor, and treatment goals are usually to prolong life and reduce pain, often by hormonal therapy and/or chemotherapy [22, 24]. In these cases where removal of the cancer is not possible,

the cancer is called inoperable, and treatment is given to shrink the cancer in the form of chemotherapy, immunotherapy, targeted drug therapy, radiation and/or hormonal therapy [25]. As mentioned under 1.1.2.1, breast cancer cells often have receptors that can bind to oestrogen and progesterone and stimulate cell proliferation. Hormonal therapy, or endocrine therapy, are treatments that prevent these hormones from attaching to the receptors, ultimately reducing the cell growth. It is given to patients with HR+ cancers, which are around 2/3 of all breast cancer cases [26]. For HER2-enriched cancers, which are HR-, targeted drug therapies are often given that target proteins on the cancer. Herceptin, also known as Trastuzumab, is one such drug that is often used in combination with chemotherapy to ultimately block the growth stimulation of the HER2 receptors [27]. Other targeted therapies may also be used for HR+ cancers and TNBC. Lastly, immunotherapy is a widely used breast cancer treatment that is aimed towards the efficiency of the patient's own immune system and its ability to recognize and destruct cancer cells.

Cancer treatment in general is challenging, and many patients experience serious side effects [23]. Chemotherapy has the possible side effects of hair loss, nausea, fatigue, and increased chance of infection and easy bruising. Negative sides of hormonal cancer treatment have been observed where some patients have developed resistance against the treatment. New approaches and methods of targeted therapy are emerging, and at the same time, a deeper understanding of the cancer and new treatment is necessary [28]. Breast cancer is a dramatically important health problem for women all over the world, and the need to understand this disease on a cellular and molecular level is important to improve its prevention and to develop and improve its therapy.

1.2 Circulating Tumour Cells

Circulating tumour cells (CTCs) are tumour cells that have separated from the primary tumour and entered the bloodstream. During the last decades, powerful new techniques have been applied to enhance our understanding of the process of cancer metastasis. Included in this research are the circulating tumour cells, which have long been assumed to be the seeds of distant metastasis. Understanding the biology and mechanisms of these cells have shown promising contributions to the early diagnosis and prevention of metastasis [29].

1.2.1 Biology of Circulating Tumour Cells

Circulating tumour cells have several distinctive biological traits that differ them from other cells in the blood, both when it comes to morphological traits like shape and

size, and biochemical traits like the presence of distinct surface markers. It is generally believed that circulating tumour cells are larger in size than most other blood cells, ranging in size from 6 µm to > 20 µm, compared to the size of blood cells that can be as small as 5 µm, but there are also large blood cells with diameters of 20 µm. CTCs are known to have a greater nuclear to cytoplasmic ratio than leukocytes, in addition to a distinct nuclear morphology. CTCs may exhibit distinct morphological characteristics depending on the primary tumour, and the cells can be very heterogeneous meaning that there is a huge variety in both the morphology and biochemical properties of the CTCs even from the same patient [30].

CTCs express proteins that distinguish them from other cells in the blood and can be used for the enrichment and detection of CTCs. The most widely used methods for detection of CTCs are the marker-dependent techniques, and the epithelial cell adhesion molecule (EpCAM) is the most widely used cell surface protein marker for positive selection of CTCs. Several types of cancer including both breast and prostate cancer have shown CTCs that are EpCAM positive [29]. Other epithelial molecular markers of CTCs include E-cadherin, claudins, and ZO-1, and other tissue specific antigens are also used as molecular markers such as HER2 [31].

The ability of CTCs to undergo epithelial-to-mesenchymal transition (EMT) is a rising area of interest as part of CTC biology. During EMT, cells of the primary tumour lose their intracellular adhesion, and acquire mesenchymal and invasive properties. Tumour cells detach themselves from the basement membrane of the epithelial layer, and enter the circulation as CTCs, able to travel to distant sites [29]. As a result of EMT, primary tumour cells acquire traits such as motility, resistance to apoptosis and immune response, and drug insensitivity. Some EMT CTCs are also observed to have stem-like characteristics like self-renewal and multilineage differentiation abilities [32]. During EMT, CTCs lose many of their epithelial characteristics by a down-regulation of epithelial markers, and an increased expression of mesenchymal markers like vimentin and N-cadherin [29].

An overview of the metastatic cascade can be seen in figure 2. The first step of metastasis is the local invasion of tumour cells, that might have undergone EMT, into surrounding tissues [29]. The now partially mesenchymal phenotype of the cells enables them to intravasate into the blood stream, which is the next step of the metastatic cascade [33]. The cancerous cells may enter the blood stream or nearby lymphatic vessels through intravasation, in which they press themselves through the wall of the blood vessels. The cancerous cells that survive the harsh environment of the circulation are then able to undergo extravasation by moving through the vessel membranes at distant sites. Extravasated cancerous cells can colonize and undergo the reversed process termed MET (mesenchymal- to-epithelial transition), which enables them to proliferate and form macro-metastases. Secondary tumours are

formed that can stimulate additional angiogenesis, supporting further growth and metastasis[29, 33].

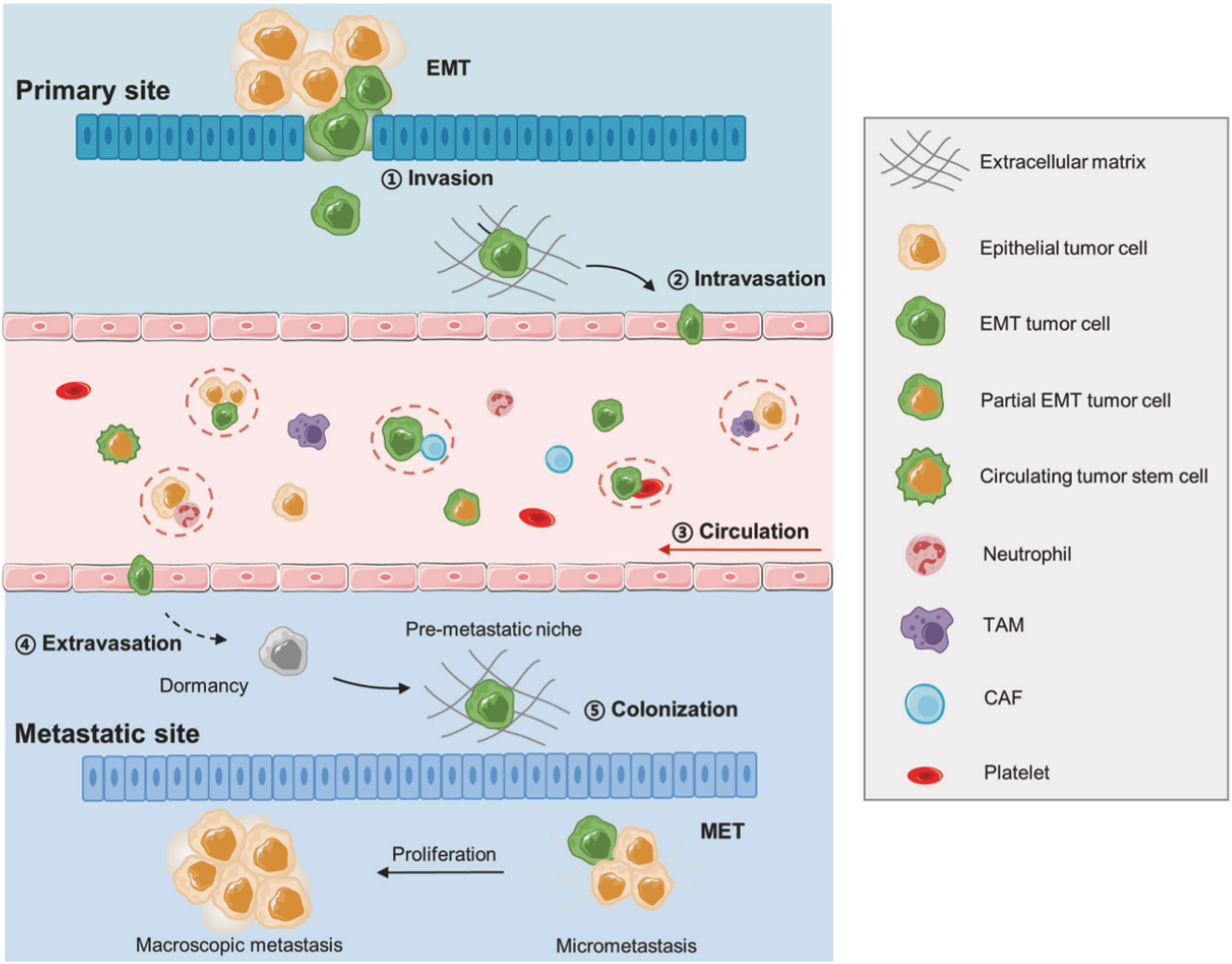

***Figure 2: The process of metastasis**. The process of metastasis includes invasion into nearby tissues, intravasation to the blood circulation, extravasation into distant tissues, micrometastasis and colonization. Reprinted from "Circulating tumour cells: biology and clinical significance" by D. Lin et al, 2021, Springer Nature, volume 6, page 3.*

Tumour cells that have entered the circulation are exposed to detrimental shear stress, anoikis and immune surveillance, and only a fraction of CTCs will survive this harsh environment [29]. CTCs have been observed to interact tightly with cells of the blood like platelets, neutrophils or cancer-associated fibroblasts (CAFs), which is believed to act as a form of protection for the tumour cells [34]. For instance, platelets have been observed bound to CTCs forming a shield-like structure, which has been found to accelerate EMT in CTCs and to promote invasion and metastasis [29]. Furthermore, CTCs have been observed to occur in CTC-clusters consisting of 2-50 circulating tumour cells, which seems to have increased ability to create distal metastasis compared to single CTCs [32], and is generally associated with worse clinical outcomes, disease progression and early mortality [35].

1.2.3 Methods for detection

Detection of CTCs can be divided into two parts. First comes an enrichment step where the CTC concentration is increased with several log units, followed by a detection steps [36]. CTC enrichment is achieved through multiple different techniques that exploit the differences between the tumour cells and other blood cells. This includes both the difference in physical properties of the cells, as well as the different expression of cell surface proteins [31].

Physical property-based techniques, also known as marker-independent techniques, utilize the physical differences between CTCs ant other blood cells, including size, densities, electrical charges and other possible deformabilities. Their larger size allows for the size-based enrichment of CTCs. Such microfiltration strategies, like the VYCAP size-based filtration technology for instance, utilize the passing of a blood sample through size-calibrated micropores to trap the large CTCs on top of the filter, and letting small blood cells pass. Another method for CTC enrichment based on physical properties is Dielectrophoresis (DEP); a technology used to detect CTCs based on distinct electrical charges of tumour cells and other blood cells [31].

The currently most used CTC detection method is the CellSearch system. The CellSearch system is the only FDA-approved clinically validated system for identification, isolation, and enumeration of CTCs from a simple blood test [37]. This system is a marker-dependent positive selection of CTCs that use ferrofluid nanoparticles with antibodies targeting the epithelial cell surface protein EpCAM. The CTCs are magnetically separated from other blood cells, followed by identification through staining with fluorescently labelled antibodies to epithelial cytokeratins, and further visualizing through fluorescence microscopy [31].

The methods above describe enrichment through positive selection – the cell of interest is targeted either through marker-independent or marker dependent approaches. There are also methods of detecting CTCs that targets the hematopoietic cells of the blood sample, known as negative selection. A drawback of positive selection methods is that a CTC may not express the chosen marker used for enrichment. With a negative selection method, non-malignant blood cells are targeted using antibodies that recognize cell surface proteins expressed on normal blood cells. There is however experiences of a lower purity of CTCs isolated using negative selection strategies, and these methods do not account for the risk that CTCs may be trapped in a mass of blood cells [31].

After enrichment, CTCs need to be detected and identified as individual cells as the sample still contains a large number of leukocytes. There are a range of different methods for detection of CTCs in a blood sample, including direct immunological

detection, isolation of CTCs by micromanipulation, or identification using nucleic-acid based strategies [31]. New research and emerging technologies are contributing to the improvement of the challenging task that is detecting the rare CTCs among the pool of normal blood cells.

1.2.4 Clinical relevance

The use of CTCs as a real-time liquid biopsy has become an interesting area of research [36], as CTCs are believed to be interesting biomarkers for a variety of solid cancers. Some clinical applications of CTCs include early detection of the disease, as well as the monitoring of the disease and treatment. CTC detection is non-invasive, which enables the monitoring of tumour progression in patients recovering from cancer [38]. Evaluating the development and prognosis of the disease by CTCs is of huge clinical relevance, and the CTCs have been shown to have prognostic value both in operable and metastatic breast cancer [39]. CTCs are used as biomarkers for monitoring the response of cancer treatment, often done in combination with imaging examination. The advantage of the non-invasive CTC detection is that it in some cases provides higher sensitivity that imaging, and radiation exposure that occurs during imaging procedures is avoided [29]. There are other applications of CTCs that are being extensively researched for future clinical application, like the understanding of CTCs role in metastasis. Metastasis is a huge obstacle when it comes to improving clinical outcomes of patients, and the role of CTCs and CTC clusters in metastatic cancer colonies has gradually gained more attention, as CTCs has commonly been assumed to be the substrate of metastasis [29].

Generally speaking, the increased number of CTCs in a patient is correlated with a higher likelihood of metastasis and cancer aggressiveness [29]. Providing significant insight into cancer metastasis, CTCs and their clinical relevance remains a huge area of research. CTC clusters have been associated with a worse prognosis in lung cancer patients, but more research on the topic is needed [36]. Lastly, using the CTCs for early detection of solid tumours have long been a topic of interest, even though there is still debate around whether or not CTCs actually disseminate from the tumour at early stages of cancer [36].

1.2 The Aim of the Project

The primary aim of this project was to establish and optimize a method for size-based CTC-enrichment in blood samples from patients with breast cancer. The aim was also to validate the methods, and to optimize the protocol with regard to blood collection tubes and immunofluorescent staining. The project was part of a larger study of CTCs in metastatic breast cancer, which aims to understand the role of CTCs in metastasis and development of treatment resistance and investigate their clinical utility in monitoring of metastatic breast cancer.

2. Material and methods

2.1 Material

2.1.1 Cell line

Two cell lines were used for this project, MCF-7 (ECACC 86012803) and ZR-75-1 (ATCC CLR1500). The MCF-7 cell line is derived from mammary gland tissue of an adenocarcinoma in a 69-year-old white woman *[40]*, and ZR-75-1 from the mammary gland tissue of a ductal carcinoma in a 63-year-old white woman *[41]*.

2.1.2 Blood samples

Blood samples were collected from seven female patients diagnosed with metastatic breast cancer. All samples were collected before patients had undergone cancer treatment, and samples were taken in the period from January to April 2023. Patients were participating in the study "Monitoring advanced breast cancer using liquid biopsies" at Stavanger university hospital (SUS). Blood samples were also derived from healthy donors to use in validation and optimization experiments. The project was approved by the regional ethical committee (REK Vest 123826) and written informed consents were obtained from all participants.

2.1.3 Reagents and equipment

A full overview of reagents used in experiments of this project are presented in table 3.

Table 3: *Reagents used for experiments. Material, manufacturer, catalogue number and application are provided.*

Material	Manufacturer	Catalogue number	Application
RPMI-1640 medium	Sigma-Aldrich	R0883-500ML	Cell culture
EMEM (EBSS) medium	Sigma-Aldrich	E7510-500ML	Cell culture
Glutamine	Sigma-Aldrich	G7513-100ML	Cell culture
Foetal Bovine Serum 10%	Sigma-Aldrich	F7524-500ML	Cell culture
Penicillin-Streptomycin 10 mg/ml	Sigma-Aldrich	P4433-100ML	Cell culture
Dulbecco´s Phosphate Buffered Saline	Sigma-Aldrich	D8537-500ML	Cell culture
Non Essential Amino Acids 1%	Thermo Fisher Scientific	111450-100ML	Cell culture
Tryptan Blue Stain	Invitrogen	T10282	Cell counting

Paraformaldehyde	Sigma-Aldrich	158127	CTC enrichment,
Trypsin-EDTA 0.25%	Sigma-Aldrich	T4049-500ML	Cell culture, splitting
Bovine Serum Albumin	Sigma-Aldrich	A7030-50G	CTC enrichment
Saponin	Sigma-Aldrich	47036-50G-F	CTC enrichment
Phosphate Buffered Saline	Sigma-Aldrich	P4417-100TAB	CTC enrichment
DAPI	Sigma-Aldrich	32670-25mG-F	IF-staining, nuclei
Anti-Pan Cytokeratin AE1/AE3 eFluor 570	Invitrogen	41-9003-82	IF-staining, keratin
Pan Cytokeratin Monoclonal antibody (C-11) PE	Invitrogen	MA5-28574	IF-staining, keratin
Anti-Hu CD45 antibody APC	Invitrogen	17-0459-42	IF-staining, APC
Anti-Hu CD61 REAdinityTM, Clone REA 761	Miltenyi Biotech	130-110-748	IF-staining, FITC
FcR Blocking Reagent human	Miltenyi Biotech	130-059-901	IF-staining
FIX&PERM permeabilization kit	Invitrogen	GAS003	CTC enrichment, fixati
ProLong Diamond Antifade Mountant	Thermo Fisher Scientific	P36965	CTC enrichment, mour

An overview of equipment used in experiments is presented in table 4.

Table 4: *Equipment used for experiments. Equipment, manufacturer, catalogue number and application provided. NA = Not available.*

Equipment	**Manufacturer**	**Catalogue number**	**Application**
Hera Cell 150 incubator	Heraeus	NA	Incubation
Hera Safe BSC	Heraeus	50073961	Cell culture
CountessTM Automated Cell Counter	Thermo Fisher Scientific	NA	Cell counting
Countess cell counting Chamber slide	Thermo Fisher Scientific	C10283	Cell counting
Olympus CKX31 microskope	NA	NA	Microscopy
Bürker chamber	Thermo Fisher Scientific	10628431	Cell counting
Micro tubes, 1.5 ml	Sarstedt	72690001	Cell counting, staining, etc.

Tube 50 ml (with cape)	Sarstedt	62559001	Cell counting, CTC enrichment
T75 Flask	Thermo Fisher Scientific	156499	Cell culture
Allegra X-30R centrifuge	Beckman CoulterTM	NA	Centrifugation
Pump	VYCAP	NA	CTC enrichment
Pump unit	VYCAP	PU-500	CTC enrichment
Microsieves	VYCAP	FS-510	CTC enrichment
Staining holder	VYCAP	SH-60	CTC enrichment
Axioplan 2 imaging microscope	NA	NA	Microscopy

An overview of blood collection tubes used in experiments is presented in table 5.

Table 5: *Blood collection tubes used in experiments. Application is also provided.*

Blood collection tube	Application
CellSave	Validation, optimalization, patient samples
EDTA	Optimalization
TransFix	Optimalization, patient samples
Streck	Optimalization

2.1.4 Prepared solutions

Some prepared solutions were used in experiments in this project and are listed in table 6.

Table 6: *An overview of prepared solutions used in experiments. Solution, protocol for preparation and application is provided.*

Solution	Protocol	Application
PBS, 0.01 M	5 tablets of PBS dissolved in 1000 ml mQ water	CTC enrichment
Saponin, 10%	5.0 g saponin dissolved in 50 ml mQ water. Slightly heated to dissolve and filtered (0.2 µm)	CTC enrichment
Paraformaldehyde, 4%	2.0 g paraformaldehyde dissolved in 50 ml PBS. Heated to 65 degrees to dissolve and filtered (0.2 µm). Dispensed in ml aliquotes and frozen	CTC enrichment, fixation
DAPI	5 mg DAPI in 1 ml mQ water	IF-staining
PBS/1% BSA	0.5 g BSA dissolved in 50 ml PBS.	CTC enrichment
PBS/0.1% saponin	10 µl 10% saponin in mQ is added to 1 ml of PBS (0.01 M).	CTC enrichment

2.2 Methods

2.2.1 Cell line culturing

The following techniques were applied to the cell lines MCF-7 and ZR75-1. Both cell lines were cultured to be used in spiking and optimization experiments.

2.2.1.1 Aseptic technique

For working with cell line culturing, aseptic technique was used to avoid contamination of the cell culture from bacteria, fungi, and mycoplasma. When working with multiple cell lines, aseptic technique also helps avoiding cross contamination of cell lines. All cell line work is performed in a cell culture room using personal protective equipment (sterile gloves, disposable laboratory coat and overshoes) and the microbiological safety cabinet. The enclosed, ventilated biosafety cabinet provides a safe working space for materials requiring a specific biosafety level. Airflow in the cabinet is filtered, removing harmful bacteria and viruses, providing a sterile and particle-free working space. Equipment, reagent bottles, and gloves are sterilised with 70% ethanol before entering the bench, and the bench itself is sterilised before use by both UV-light and 70% ethanol.

2.2.1.2 Resuscitation of Cell Line

Cell line cells are stored in a nitrogen tank at -196°C and must be thawed and put into culture. It is essential that cultures are thawed quickly, and rapidly diluted in culture medium.

Medium formulation used for the MCF-7 cell line:

- Minimum Essential Medium Eagle
- 10% Foetal Bovine Serum
- Penicillin-Streptomycin 10mg/ml
- 1% Non-Essential Amino Acids
- 2 mM Glutamine

Medium formulation used for ZR-75-1 cell line:

- RPMI 1640
- 10% Foetal Bovine Serum
- 200 mM Glutamine
- Penicillin-Streptomycin 10mg/ml

Protocol 1: Resuscitation of cell line

1. Preheat the medium for the particular cell line cells to 37 °C.
2. Turn on the UV-light in the sterile bench for minimum 30 minutes prior to use.
3. Sterilize the bench, medium bottle, ant gloves with 70% ethanol before entering the sterile bench. Always use aseptic technique in the sterile bench.
4. Transfer 20 ml preheated medium to a T75 bottle. Mark bottle with cell type, date, and cell subculture generation.
5. Take up an ampoule of cells from the -196°C nitrogen storage tank and thaw quickly by placing the ampoule in a 37 °C. water bath. Take the ampoule away from the water bath when there is still a small lump of ice left in the tube. Move on quickly to the next step.
6. Wipe the ampoule with 70% ethanol before entering the sterile bench. Transfer the whole content of the tube to the T75 flask carefully with a 2 ml pipette. Mix gently by turning the flask.
7. Incubate at 37 °C with 5% CO_2 until cells need to be split (usually around 2-3 days).

2.2.1.3 Subculturing of Adherent Cell Lines

Both MCF-7 and ZR-75-1 are adherent cell lines that grow relatively quickly and need to be subcultured in order to prevent them from dying. The cell line cells of this experiment attach to the bottom of the flask and need to be brought into suspension in order to be subcultured.

Protocol 2: Subculture of adherent cell lines

1. Preheat medium, PBS (without Ca^{2+}/Mg^{2+}) and trypsin EDTA to 37°C for 30 minutes. Turn on the UV-light in the sterile bench 30 minutes before use.
2. Observe cells under the microscope to determine degree of confluence, as well as determining absence of contamination. Decide on amount of medium and trypsin-EDTA that should be used for the splitting.
3. Use aseptic technique, and make sure to sterilize bench, bottles, and gloves with 70% ethanol before entering sterile bench. T75 bottles should not be sterilized with ethanol.
4. Carefully remove medium with a 25 ml pipette and discard.
5. Wash bottom of the flask with 10 ml preheated PBS. Add PBS carefully and rinse the flask by rotating the flask. Remove PBS with the same pipette, and discard.
6. Add the suitable amount of trypsin-EDTA, determined in step 1 (ideal amount is 1 ml per 25 cm^2). Rotate the flask to cover the whole bottom.
7. Incubate at 37°C for 5 minutes in a 5% CO_2 incubator.

8. Observe the flask under light, or under microscope, to determine whether all cells have detached from the bottom. If they are still attached, incubate for 30sek-1min longer.
9. Add an excess of medium to the T75 flask. Mix well with the pipette by pressing suspension against the bottom of the flask 5-10 times.
10. Prepare a new T75 flask by adding 20 ml medium, then transfer a suiting volume (determined in step 1) of cell suspension to the new flask. Mix by rotating flask.
11. Observe cells under the microscope to determine whether splitting was successful.
12. Incubate at 37°C in 5% CO_2 incubator until next splitting.

2.2.2 Cell Counting

Cell counting was used for spiking experiments to determine volume of desired cells.

Protocol 3: Cell counting with Countess™ automated cell counter

1. Turn on the Countess™ automated cell counter and choose the appropriate setting.
    a. For counting of cell line, choose “Cell line”.
    b. For counting of PBMC, choose “PBMC”.
2. Stain cells with tryptan blue.
    a. For staining cancer cells: Stain cancer cells with Tryptan blue in a 1:1 ratio (50:50 µl) in a 1.5 ml Eppendorf tube.
    b. For staining PBMC: Prepare a solution with 45 µl PBS + 5 µl cell suspension + 50 µl 0.4% Tryptan blue in a 1.5 ml Eppendorf tube.
3. Transfer a small amount (~10 µl) of the solution to a Countess™ chamber slide and let it rest for 30 seconds.
4. After 30 seconds, insert the slide into the cell counter machine.
5. Start count by pressing capture. Countess™ automated cell counter provides concentration of alive and dead cells.

Protocol 4: Manual cell counting with visible light microscope

1. Centrifuge the cell suspension at 300*g for 5 minutes (if necessary). Remove the supernatant and resuspended the pellet in PBS/1%BSA, volume dependent on degree resuspension required.
2. Stain the cell suspension 1:1 with tryptan blue (50 µl of each) in an Eppendorf tube and mix by pipetting up and down a few times.
3. Transfer a small amount (~20 µl) of the suspension/tryptan blue mix under the cover-slide glass of a Bürker Counting Chamber.

4. Count full 4x4 squares cells in an established pattern (see figure 3) until at least 200 cells are counted (if possible).

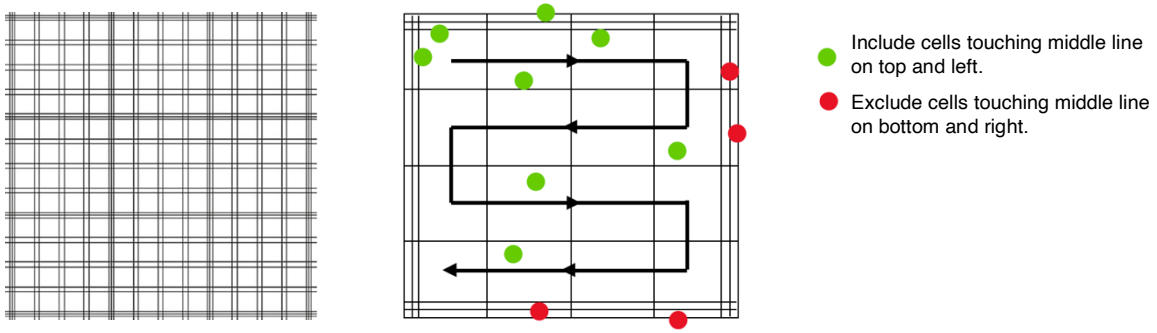

Figure 3: Established counting pattern. *Pattern is used in manual cell counting using the Bürker counting chamber. Left: Whole Bürker chamber. Right: Emphasized 1/9 squares of Bürker chamber. Squares are counted individually in pattern established by the arrow until 200 cells are counted (if possible).*

5. Calculate the cell concentration using the following equation I.

$$I.\ CELLS\ PER\ ml = AVERAGE\ CELLS\ PER\ 4x4\ SQUARE * DILUTION\ FACTOR * 10\ 000$$

6. Calculate the volume cell suspension corresponding the desired number of cells for use in the spiking experiment using the following equation II.

$$II.\ CELL\ SUSPENSION\ (\mu l) = \frac{NUMBER\ OF\ CELLS\ DESIRED}{CELL\ CONCENTRATION} * 1000$$

2.2.3 Cell Line Spiking

Spiking of tumour cell line cells into healthy blood samples was done to validate and optimize the method of size-based filtration. The protocol was applied to both the MCF-7 and ZR-75-1 cell line.

Protocol 5: Spiking of cell line cells into healthy blood samples

1. Collect a blood sample from a healthy individual in the blood collection tube (BCT) of choice.
2. Remove the lid of the BCT and transfer a volume of cell suspension corresponding to a fixed amount of tumour cell line cells into the blood sample with a pipette. Make sure all suspension is transferred by pipetting up and down a few times. Place the lid back on.
3. Mix well by inverting the BCT 4-5 times.
4. Incubate the BCT at room temperature for 24 hours (or incubation time of choice) before proceeding to the size-based enrichment analysis.

2.2.4 CTC Enrichment

Size-based CTC enrichment is applied to validation experiments, optimization experiments and in patient sample analyses. In this project, CTCs are enumerated and detected using the VYCAP size-based filtration system.

2.2.4.1 VYCAP

Protocol 6: VYCAP size-based filtration and immunofluorescent staining of cells

Part 1: Filtration

1. Set the VYCAP filtration pump to a suitable pressure. Ideal filtration speed is such that it takes 1-3 minutes to filter 1 ml blood. Different test tubes may require different pressures.
2. Insert a new filtration unit into the pump, and make sure the o-ring and the filter unit is completely intact.
3. Mix the blood sample well by inversion and transfer the entire sample to the sample side of the filtration unit with a pipette.
4. Turn on the pump. Swich off just before the entire sample has passed, making sure not to leave the pump on once sample has passed.
5. Wash blood sample tube with 1ml PBS/1% BSA and transfer the liquid to the sample side of the filtration unit to wash away remaining blood from around the edges of the filtration unit tubes.
6. Turn on the pump. Swich off just before the entire washing solution has passed, making sure not to leave the pump on once liquid has passed.
7. Repeat washing step by adding 1ml PBS/1% BSA directly to the filtration unit.
8. Remove the microsieve slide from the filtration unit and place in a staining holder with an absorber.

Part 2: Staining

9. Gently press down the microsieve slide against the absorber to remove remaining fluid. In cases where not all the liquid is removed, the corner of a tissue may be used by putting the tip in one of the corners of the microsieve. Release the pressure to disconnect contact with the absorber.
10. Wash the microsieve by pipetting 75 µl PBS/1% BSA gently to the centre of the sieve. Push the sieve down against the absorber to remove washing liquid, and release pressure to disconnect contact with absorber.
11. Repeat step 10.

12. **Fixation**:
    a. For fixation with 4% paraformaldehyde:
        i. Gently pipette 100 µl 4% paraformaldehyde to the centre of the microsieve. Incubate at room temperature for 20 minutes.
    b. For fixation with FIX&PERM fixation solution:
        i. Gently pipette 100 µl of Component A of the FIX&PERM reagents to the centre of the microsieve. Incubate at room temperature for 20 minutes.
13. Remove fixation solution by pressing down against the absorber, and then release pressure.
14. Wash the microsieve once with 75 µl PBS/1% BSA and remove washing solution by pressing down against the absorber, and then release pressure.
15. Add 53,3 µl pre-made antibody mixture to the filter and incubate 20 min at room temperature with no light. Table 2.5 below shows the different antibody mixtures used for the different fixation techniques. Multiply volumes to make the master mix.

Table 7: *Components of antibody mixtures used in immunofluorescent staining of cells. Reagents and amounts are provided for each of the fixation methods Paraformaldehyde and FIX&PERM.*

Fixation with 4% paraformaldehyde		**Fixation with FIX&PERM**	
Reagent	**Amount (µl)**	**Reagent**	**Amount (µl)**
PBS/0.1% saponin	40	FIX&PERM Component B	50
FcR blockage solution	10	0.1 mg/ml DAPI	0.5
0.1 mg/ml DAPI	0.5	Anti-Pan Cytokeratin AE1/AE3 eFluor 570	0.5
Anti-Pan Cytokeratin AE1/AE3 eFluor 570	0.5	Pan Cytokeratin Monoclonal antibody (C-11) PE	0.5
Pan Cytokeratin Monoclonal antibody (C-11) PE	0.5	Anti-Hu CD45 antibody APC	1
Anti-Hu CD45 antibody APC	1	Anti-Hu CD61	1
Anti-Hu CD61	1		
Total	***53.5***		***53.5***

16. Remove antibody mixture by pressing down against the absorber, and then release the pressure.
17. Wash the microsieve once by pipetting 75 µl PBS/1% BSA gently to the centre of the sieve. Push the sieve against the absorber to remove washing liquid, and release pressure to disconnect contact with absorber.
18. Apply 75 µl PBS/1% BSA gently to the centre of the sieve again, and this time incubate with the washing solution for 5 minutes at room temperature without light before removing the liquid.
19. Press extra hard against the absorber until the microsieve looks dry. Incubate the sieve for 10 minutes at room temperature without light to let the filter air dry.
20. After air drying, turn sieve upside down in microsieve holder and add 15 µl mounting solution to the backside of the sieve.

21. Apply a VYCAP cover glass with tweezers on top of the mounting solution, avoiding bubbles. Do not press on cover glass after mounting.
22. Turn the sieve right sides up in the microsieve holder and add 13 µl mounting solution to the front side of the sieve.
23. Apply a VYCAP cover glass with tweezers on top of the mounting solution, avoiding bubbles. Do not press on the cover glass after mounting.
24. Incubate the sieve for 10 minutes in room temperature without light, and then acquire images.

2.2.4.2 Immunofluorescence Microscopy

Immunofluorescent staining was used in this project to visualize and distinguish between cells. This was achieved through the combination of specific antibodies tagged with fluorophores that bind to the specific antigens on the target cell [42]. Immunofluorescent staining is used in both validation and optimization experiments to distinguish between blood cells and cell line cells, and in patient samples to distinguish between blood cells and CTCs. Blood cells (leukocytes) were stained red with antibodies against CD45, cancer cells were stained yellow with antibodies against keratin, and all cell nuclei were stained blue with DAPI. Platelets were also stained using CD61 antibody (green fluorescence).

A Leica Axioplan 2 fluorescence microscope was used in this project. All molecules have the ability to absorb light of a specific wavelength. When a sample is illuminated with light of a specific wavelength from the microscope, any fluorophore in the sample will absorb the light wavelength and become excited to a higher energy state. As the fluorophores return to their original energy state, they release the excess energy in the form of emitted light of a longer wavelength which may be detected using the fluorescence microscope (see figure 4) [42]. To detect the specific fluorescent signals from the sample, the fluorescent microscope uses filters to separate the excitation and emission wavelengths of the fluorophores. The most energy-rich light is absorbed by the fluorophores, and the other light is absorbed by the filters. This means one can get a visual representation of the location and distribution of the fluorescent molecules in the sample [42].

For this project, four different fluorophores were used. It is important to avoid spectral overlap when choosing fluorophores. Figure 4 illustrates the different fluorophores and corresponding emission and excitation wavelengths. Some spectral overlap is observed for both emission and excitation wavelengths. The specific filters used allows observation of the specific fluorescent antibodies with little cross signals from other wavelengths. For an overview of the specific fluorophores and filters used, see appendix A.

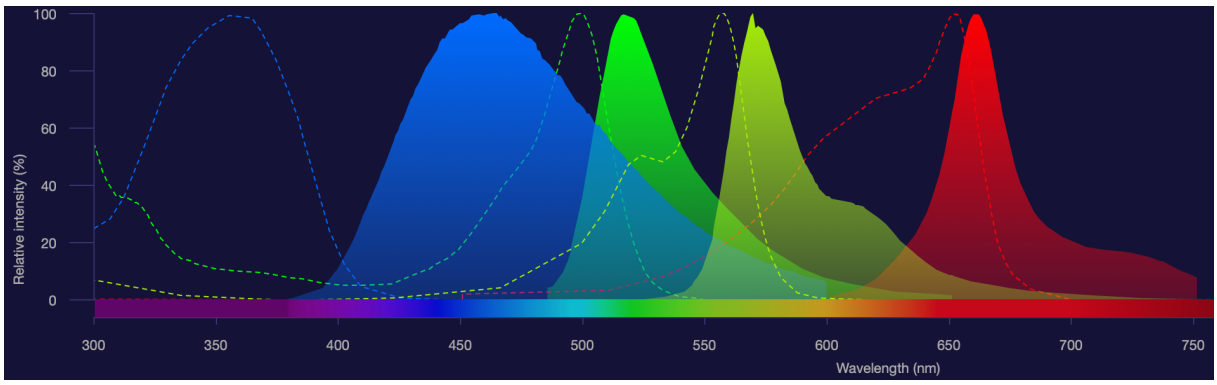

***Figure 4: Excitation and emission of fluorophores**. The four fluorophores used for this experiment was DAPI (blue), FITC (green), eFluor 570 (yellow), and APC (red). Filled in graph: Emission. Dotted graph: Excitation. Reprinted from ThermoFishers "Spectraviewer" https://www.thermofisher.com/order/fluorescence-spectraviewer.*

Microsieves with samples were observed manually and scanned in Metafer 4 (version 3.5.12) as well as taking pictures in ISIS (version 5.2.23). Scanning with Metafer was done using the 10x objective. Pictures taken with ISIS are done by the 10x and 40x objective.

Protocol 7: Scanning in Metafer 4

5. Open Metafer and use RCDetect.
6. Use the Classifier Setup (CTC2022). The following settings were used for the colour channels:

Colour channel	Exposure mode	Integration time (seconds)
Blue	Fixed	0.0417
Green	Fixed	0.6250
Red	Fixed	4.7917

7. Place the microsieves in the slide holder, and place this under the microscope.
8. In Metafer, click Setup, and enter the name of the sample. Use Mode = RCD, Classifier = CTC2022, Search Window = Manual, Max. Cnt = 10 000. Click OK.
9. Click Search and determine the area of the slide that is to be scanned. Click OK and scan the sample.
10. For analysing the sample and counting CTCs, click Training, and Classify fields. Choose the sample you wish to analyse and start counting.

2.2.4.3 Classification of CTCs

Classification of CTCs is applied in all immunofluorescence microscopy analyses. In validation and optimization experiments, cell line cells are classified, and in patient samples CTCs and CTC clusters are classified.

Protocol 8: Identifying CTCs

1. Prepare the sample though established procedures, and scan using Metafer.
2. Consider the keratin stain (green): Look for cells that are strongly positive for keratin. Cancer cells should be positive for keratin.
3. Consider CD45-stain (red): Determine whether the cell is positive for CD45. Cancer cells should not be positive for CD45.
4. Consider DAPI stain (blue): Determine whether the cell nucleus is stained with DAPI. All cell nuclei should be stained with DAPI.
5. Consider the morphology: Cancer cell line cells are generally larger than leukocytes and have a greater nuclear to cytoplasmic ratio. For analysing patient samples, smaller cells must be considered more carefully. Cancer cells should also be mononuclear. All cells should have a distinctly round shape.
6. If a cell is clearly keratin-positive, CD45-negative, and has a DAPI stained nucleus in addition to being mononuclear, classify as a CTC.
7. If a cell is weakly keratin-positive, has very low CD45, and has a DAPI stained nucleus in addition to being mononuclear, classify as a potential CTC.

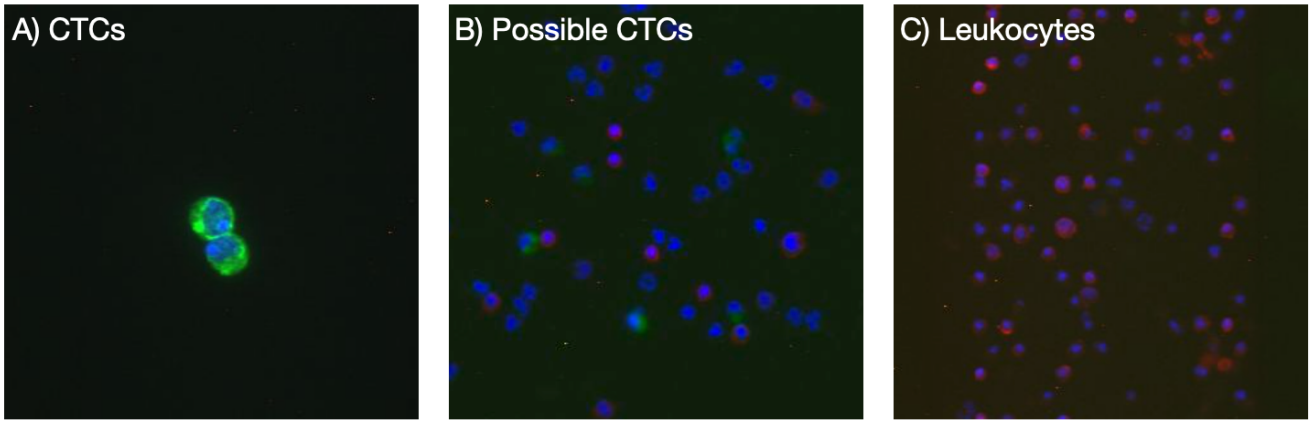

Figure 5: Classification fractions of CTCs, possible CTCs and leukocytes. *The cells were stained with anti-keratin antibodies (green), anti-CD45 antibody(red) and DAPI (blue). A) shows a fraction of a spiked-in sample with ZR-75-1 cell line cells. B) shows a fraction of weakly keratin-stained, weakly CD45-stained cells of a patient sample. C) shows leukocytes of a negative control sample. Pictures are taken in the Metafer software using 10x objective.*

3. Results

The results from this project can be divided into three parts – validation (1), optimization (2) of the CTC enrichment and detection method, and analysis of patient samples (3). The VYCAP size-based filtration protocol was validated prior to being applied to patient samples. After validation, the protocol was optimized by determining the most optimal blood collection tube and permeabilization combination. An overview of the experimental approach is shown in figure 6.

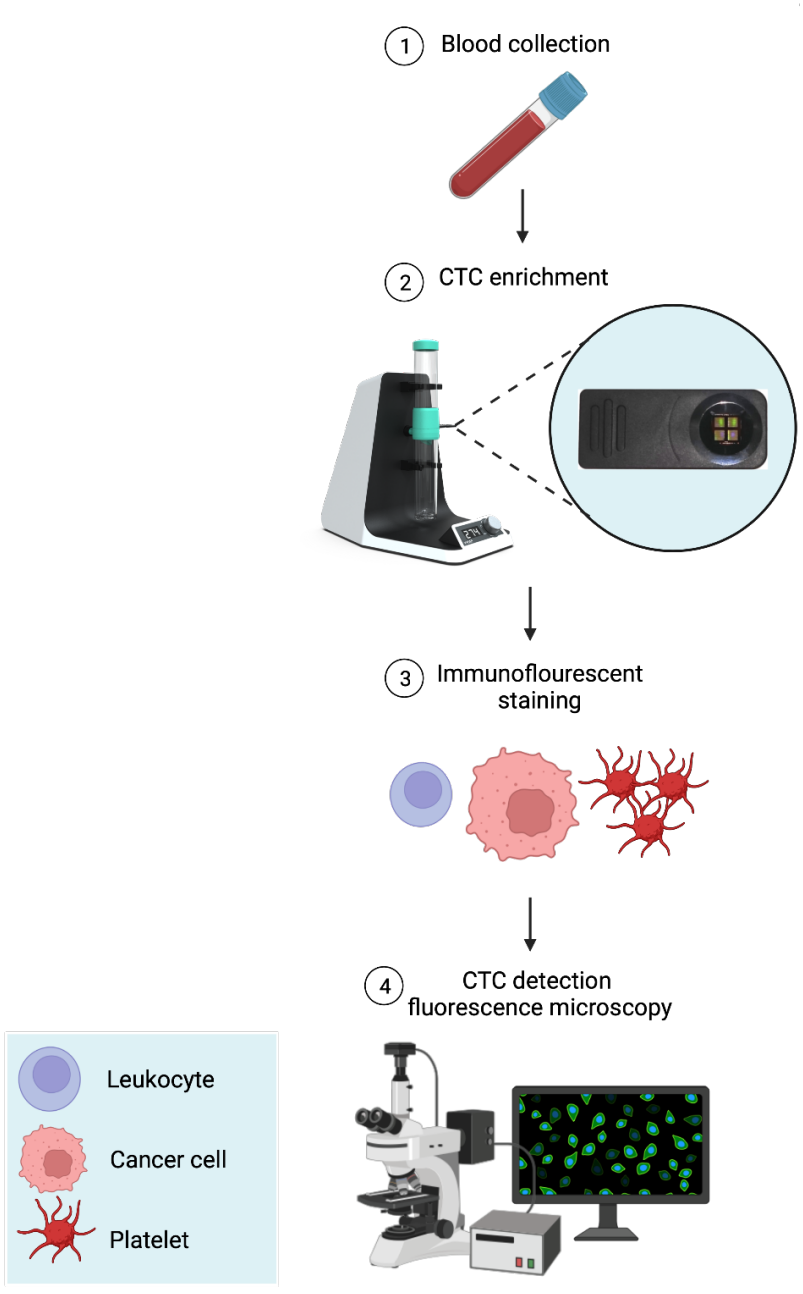

Figure 6: Workflow of experimental approaches. *Workflow numbered 1-4 is the main experimental approach that has been validated, optimized and ultimately applied to patient samples. Figure created with https://app.biorender.com.*

3.1 Validation of the VYCAP System for Size-Based CTC-Enrichment

Validation of the VYCAP size-based CTC enrichment procedure was done to determine the tumour cell recovery and purification efficiency of the approach. Validation was done by spiking normal blood samples from healthy volunteers with cancer cell line cells.

We performed several validation experiments, spiking blood samples from healthy controls with either MCF-7 cell line cells or ZR-75-1 cell line cells. Validation experiments started out using the MCF-7 cell line, due to their large size and distinct morphology. A decision was made to resuscitate the ZR-75-1 cell line to use in further spiking experiments because this cell line had already been tested out with the VYCAP method, and we would be able to obtain more comparable results with previous experiments.

All validation experiments were done using CellSave blood collection tubes, which contain a fixative. It was decided not to use unfixated blood as results from previous experiments showed that cells were more damaged and less intact when using unfixated blood in the VYCAP filtration protocol [43]. Spiking experiments, tumour cell enrichment and immunofluorescent staining were performed according to protocols 5 and 6. The validation experiments commenced by introducing 200 or 1000 tumour cell line cells into blood samples from a healthy control person. The spiked blood samples were incubated at room temperature for 1 or 24 hours. Previous experiments with the ZR-75-1 cell line indicated that a 24-hour incubation was the optimal duration [43], and it was decided to use this as the standard incubation time in all spiking experiments. .

The mean recovery rate for the MCF-7 cell line was 16% average, as presented in table 8. Means of lowering the pressure of the filtration was applied to increase the recovery rate. The automated cell counting method was also carefully investigated, with a conclusion being drawn that the number of counted cells was too low to obtain reliable cell concentration numbers. It was concluded to use manual cell counting and upconcentration of cells by centrifugation to enhance the estimation of cell concentration (see protocol 4). The average recovery percentage of the ZR-75-1 cell line using the VYCAP size-based enrichment was 27%. Even though this was better than the 16% average recovery of MCF-7 cell line cells, it was still lower compared to previous experiments with the same cell line where average recovery was 48,7% [43]. For a more detailed overview of all the performed validation experiments, see Appendix B1.

Table 8: *Overview of recovery of cell line cells MCF-7 and ZR-75-1. Healthy blood samples taken in CellSave blood collection tubes were spiked with a fixed amount of cell line cells. Cells were counted using fluorescence microscopy after filtration with the VYCAP technique. NC = not counted*

Cell line	Sample number	Number of cells spiked	Number of cells recovered	Recovery percentage
MCF-7	Validation 1	200	34	17,0 %
	Validation 2	200	13	6,5 %
	Validation 3	1000	200	20,0 %
	Validation 4	1000	186	18,6 %
	Average			***16 ± 6%***
ZR-75-1	Validation 5	1000	NC	NC
	Validation 6	1000	NC	NC
	Validation 7	1000	292	29,2 %
	Validation 8	1000	NC	NC
	Validation 9	1000	162	16,2 %
	Validation 10	1000	304	30,4 %
	Average			***27 ± 8%***

3.2 Validation of Immunofluorescent Staining of Tumour and Blood Cells

3.2.1 Immunofluorescent Staining of Cells

Size-based enrichment by the VYCAP system was followed by immunofluorescent staining to distinguish between the cells of the sample. Cell line cells and cancer cells were stained with antibodies against keratin (green). Leukocytes were stained with antibodies against CD45 (red), and all cell nuclei were stained with DAPI (blue). The immunofluorescent staining technique was successful, as it allowed for the differentiation between the cells, enabling their enumeration, as depicted in figure 7 below. Platelets were stained with antibodies against CD61, and although they were a part of the study, limited attention was given to their presence during analysis.

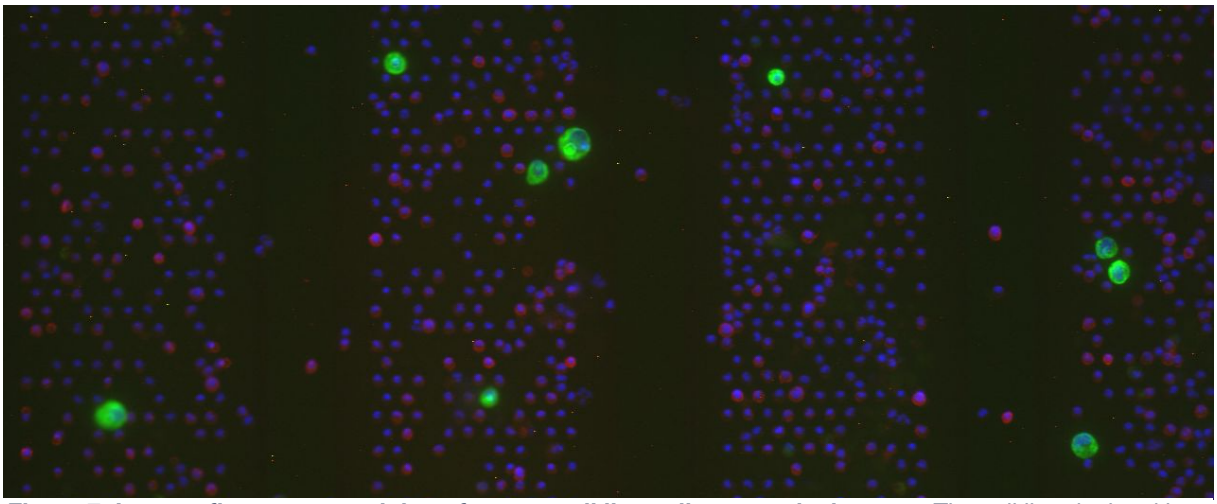

Figure 7: Immunofluorescent staining of cancer cell line cells versus leukocytes. *The cell line depicted in this figure is the ZR-75-1 cell line, stained with antibodies against keratin (green). Leukocytes are stained with antibody against CD45 (red). All cell nuclei are stained blue with DAPI. Picture taken using the Metafer 4 software and 10x objective.*

In addition to spiked-in samples, negative control samples obtained from healthy individuals were also analysed. There were observed cells that were weakly positive for both CD45 and keratin. These cells were found in all negative control samples, as well as in the spiked-in samples of validation experiments. Most likely, these cells are therefore also present in patient samples, which proposes a possibility for false positive CTCs when analysing the patient samples. For examples of these cells, see Appendix B2.

3.2.2 Immunofluorescent Staining of Cell Line Cells Without Filtration

Due to the continuing problems of low recovery percentage, two validation experiments were performed to rule out the possibility that the staining procedure was the source of low recovery rates. A volume corresponding to 1000 cell line cells was diluted in 50 µl PBS and pipetted directly on to the microsieve filter. Protocol 6 was then followed as usual from step 10. Immunofluorescent staining, scanning in metafer and classification of cancer cells was performed according to protocols 6, 7 and 8. A recovery rate of 97,8% was observed (see table 9), suggesting that low recovery rates were a result of some preceding steps, and not the staining procedure itself.

Table 9: *Overview of recovery of the cell line ZR-75-1 without filtration. 1000 ZR-75-1 cells were applied directly on to a microsieve filter, and subsequently fixated and stained. Cells were counted with fluorescence microscopy.*

Cell line	Parallel	Amount of cells applied	Amount of cells recovered (average)	Recovery percentage (average)
ZR-75-1	1	1000	996	99,6 %
	2	1000	960	96,0 %
Average			***978***	***97,8 %***

In both parallels 1 and 2, cells stained well, with big nuclei stained with DAPI (blue) and uniform keratin staining (green). None of the cancer cells were positive for CD45. There were however examples of cells in both parallels where cells were very weakly keratin stained, and some cells had no observable keratin stain at all. The amount of these cells present in validation samples was low and most likely did not influence the recovery percentage. Staining was therefore ruled out as being the source of the low recovery rates. (For examples of these cells, see Appendix B5).

3.2 Optimization of Blood Collection Tube, Fixation and Permeabilization

3.2.1 Optimization of Blood Collection Tube

Collecting blood from patients is the first step in CTC enrichment, and different blood collection tubes were tested to evaluate which tube is most optimal for size-based filtration. An overview of the experimental approach can be found in figure 8.

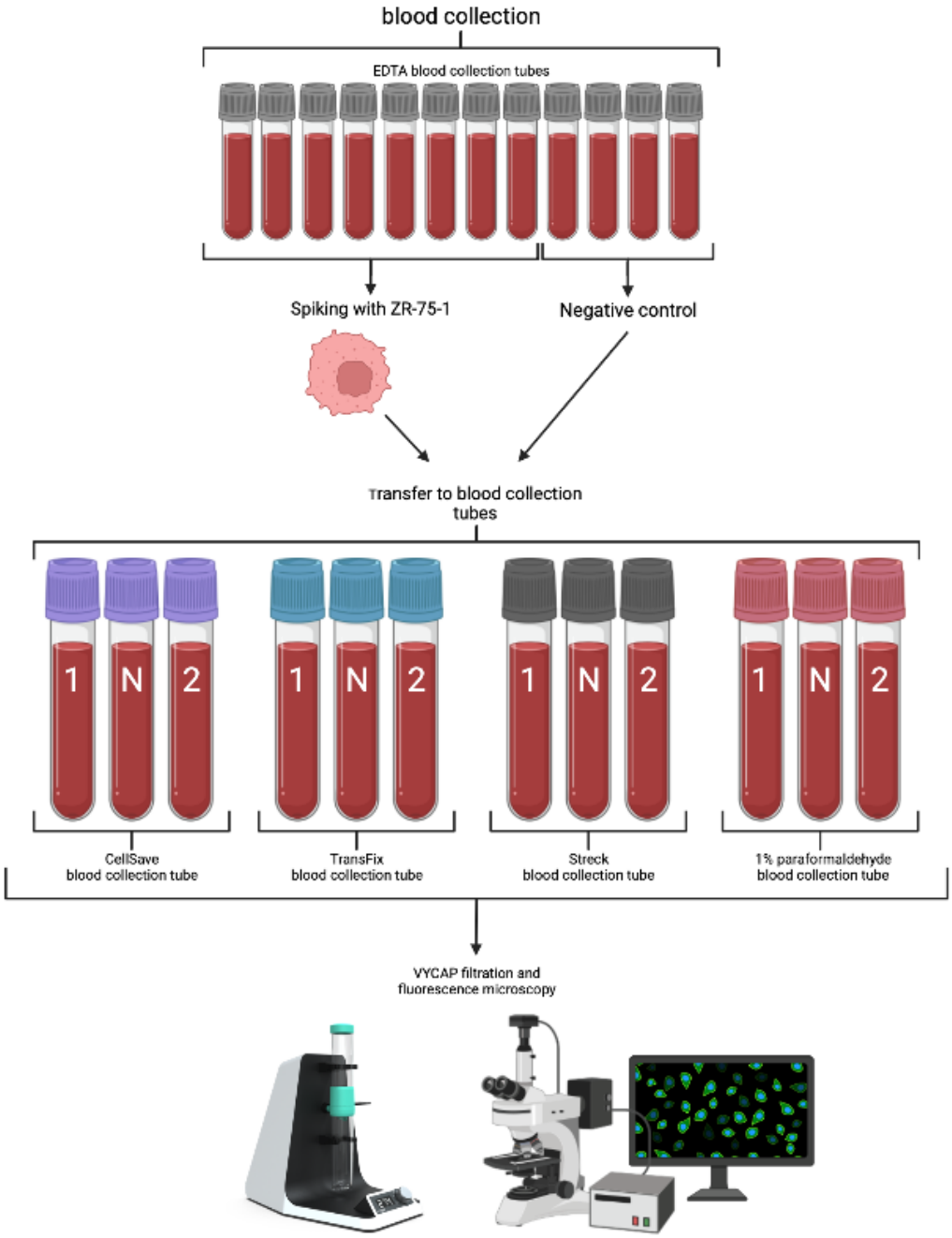

Figure 8: Overview of the experimental approach to blood collection tube optimization. VYCAP size- based filtration was used I all experiments, and samples were analysed using fluorescence microscopy. N = negative control sample. Figure created with https://app.biorender.com.

The blood collection tubes tested and compared with each other in this experiment were CellSave, TransFix, Streck and an EDTA tube with paraformaldehyde added to a final concentration of 1%. TransFix tubes are the tubes recommended in the VYCAP protocol [44]. The experiment was performed by collecting 12 blood samples from a healthy volunteer in EDTA blood collection tubes (figure 8). Eight of these samples were spiked with 1000 ZR-75-1 cells counted using protocol 4, and four samples were reserved for negative controls. Following spiking, EDTA-tubes were incubated for one hour with cell line cells, inverting tubes every 5 minutes. Using EDTA tubes initially for all samples gave all parallels the same starting point of interaction between tumour cells and blood cells. Then the blood samples (with or without tumour cell lines) were transferred to other blood collection tubes according to figure 8. For each of the four types of tubes, three samples were made; one negative control with no tumour cells, and two replicates with 1000 ZR-75-1 cells. All samples were analysed according to protocol 5, and subsequently analysed by fluorescence microscopy. The barplot in figure 9 presents the results of recovered cells for the four blood collection tubes tested. For a detailed overview of the optimization experiments of blood collection tubes, see appendix B3.

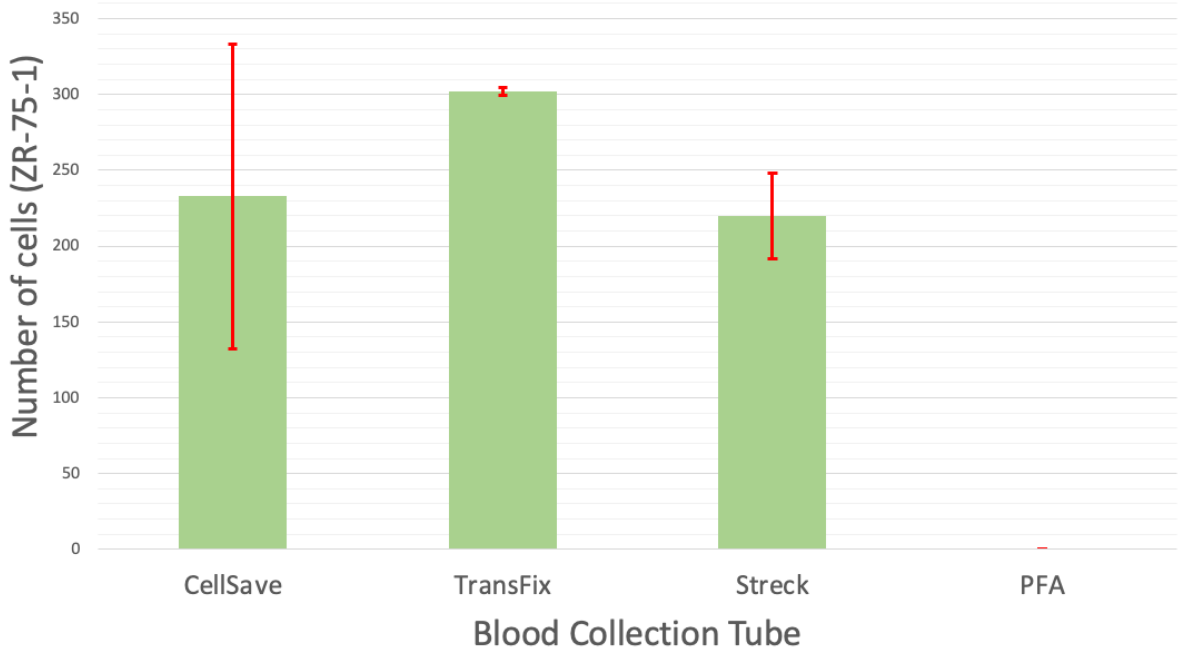

***Figure 9: Barplot illustrating recovered cells in optimization of blood collection tube**. Four blood collection tubes CellSave, TransFix, Streck and PFA were tested. All blood samples were derived from healthy individuals and spiked with 1000 ZR-75-1 cell line cells. The barplot displays total number of cells recovered (green). Error bars show the standard deviations. PFA tubes were not enumerated. PFA=paraformaldehyde.*

Figure 9 shows the number of recovered cells for each of the blood collection tubes tested, in addition to the standard deviation for each tube. The average recovery percentage for each of the tubes CellSave, TransFix and Streck was 23.3%, 30.2% and 22.0%, respectively. The standard deviation for the CellSave blood collection tube is relatively large compared to the other tubes, making it hard to determine

whether the other tubes are significantly more optimal. When filtrating the 1% paraformaldehyde tubes, the sample could not be filtered through, even when the pressure was increased to 200 mbar. When inspecting the microsieve, we observed that blood had coagulated on top of the filter, making it impossible for the rest of the sample to pass through. Clots could not be observed in the test tube before transferring the sample to the filtration unit, and the same phenomenon happened to all samples. These samples were therefore not analysed by fluorescence microscopy.

Microscopy analysis of samples from Steck blood collection tubes showed less intact ZR-75-1 cells. Many of these cells had strange shapes (not round), and many appeared to be smudged out against the background of the filter. This was the case for all Streck samples. Examples of cells are presented in figure 10 below.

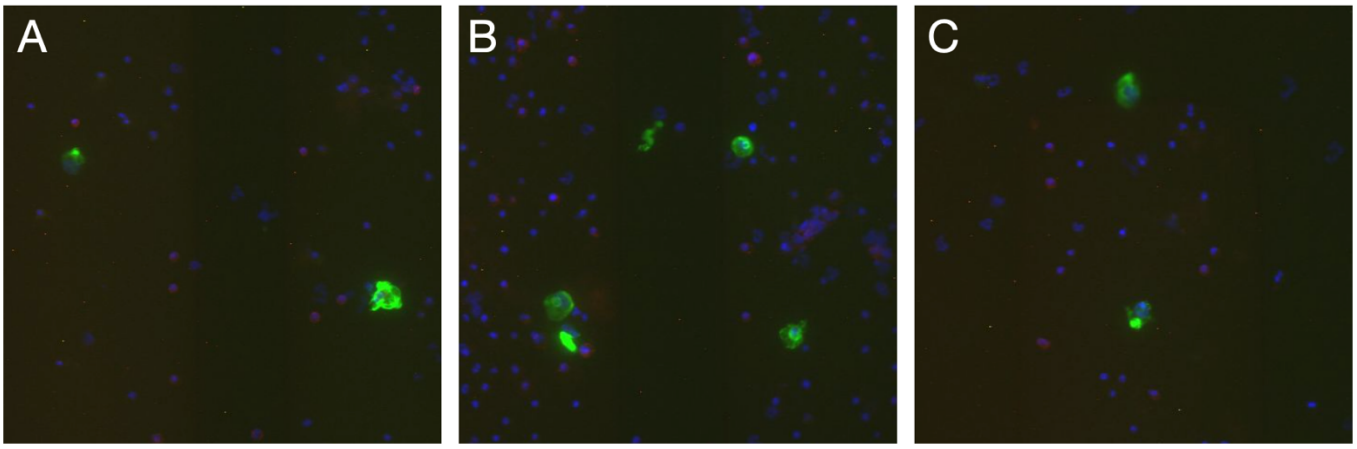

Figure 10: Immune fluorescence microscopy analysis of enriched cell fraction using Streck blood collection tubes. *The cells were stained with anti-keratin antibodies (green), anti-CD45 antibody(red) and DAPI (blue). A, B and C show fractions of cells from spiked-in samples with ZR-75-1 cell line cells after VYCAP size-based filtration. Pictures are taken in the Metafer software using 10x objective.*

CellSave tubes were used for most of the validation experiments. Optimization results with CellSave tubes gave an average recovery percentage of 23.3%, with a standard deviation of 10,04%. Compared to validation experiments with the same blood collection tube and cell line where the average was 27% (see table 8), results show reproducibility. Cells appeared generally intact; most cells were round and did not look deformed, as they did in the Streck samples. Relatively few blood cells were observed, and some staining of the background which made distinguishing the cancer cells a challenge in certain areas. Examples of cancer cells from CellSave blood collection tubes are presented in figure 11 below.

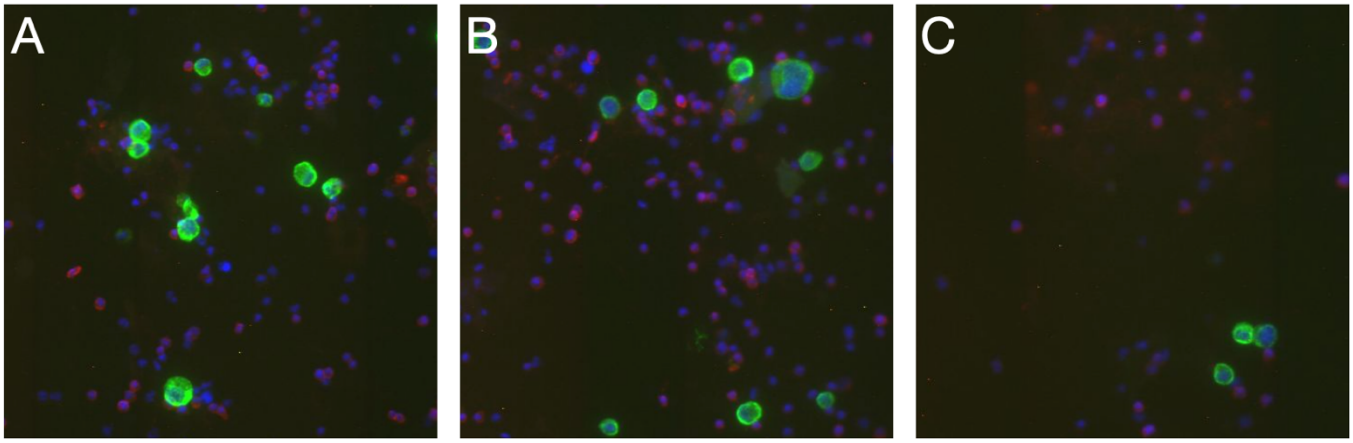

***Figure 11: Immune fluorescence microscopy analysis of enriched fraction using CellSave blood collection tube**. The cells were stained with anti-keratin antibodies (green), anti-CD45 antibody(red) and DAPI (blue). A, B and C show fractions of cells from spiked-in samples with ZR-75-1 cell line cells after VYCAP size-based filtration. Pictures are taken in the Metafer software using 10x objective.*

Lastly, TransFix blood collection tubes gave an average recovery percentage of 30.3%. Microscopy revealed numerous blood cells, and an organized fix of cells in microwells which was not observed in the other test tubes to the same extent. More small cancer cells appeared to be present compared to the other blood collection tubes, which may indicate that less small cancer cells are lost during filtration. Examples are presented in figure 12 below. Furthermore, cells appear to be intact, with round shapes and even, prominent staining.

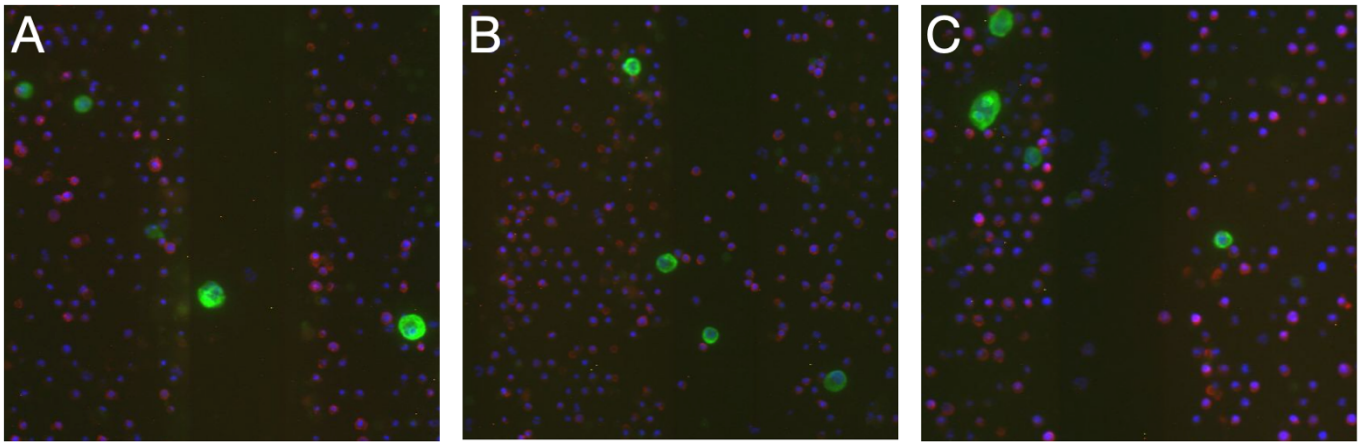

***Figure 12: Immune fluorescence microscopy analysis of enriched fraction using TransFix blood collection tube**. The cells were stained with anti-keratin antibodies (green), anti-CD45 antibody(red) and DAPI (blue). A, B and C show fractions of cells from spiked-in samples with ZR-75-1 cell line cells after VYCAP size-based filtration. Pictures are taken in the Metafer software using 10x objective.*

Comparing all three of the analysed blood collection tubes Streck, CellSave and TransFix, TransFix tubes have the best recovery percentage. It is hard to determine whether TransFix actually is significantly better than the two others due to the huge difference in standard deviations. Determining TransFix as the most optimal blood collection tube for this procedure is therefore primarily based on the recommendation from VYCAP, in addition to the numerous intact, well stained cells observed during fluorescence microscopy.

3.2.2 Optimization of Fixation and Permeabilization

Permeabilization is a crucial step in the VYCAP size-based filtration protocol because it makes it possible for the fluorescent antibodies to access the cell interior. An overview of the experimental approach can be found in figure 13.

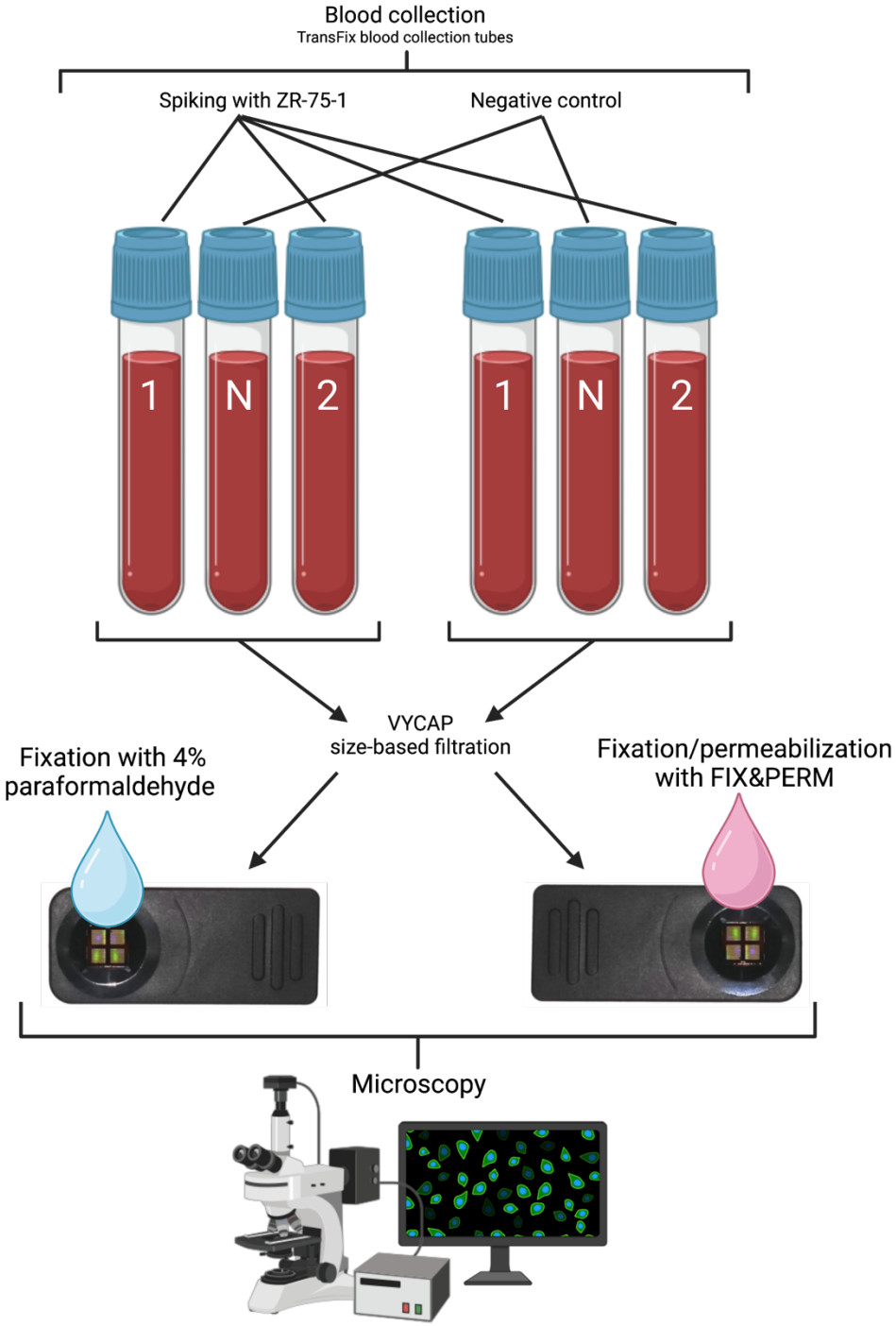

Figure 13: Overview of the experimental approach to optimization of permeabilization and fixation. VYCAP size- based filtration was used in all experiments, and samples were analysed using fluorescence microscopy. Figure created with https://app.biorender.com.

Optimization experiments were performed with both paraformaldehyde and the FIX&PERM kit to determine the most optimal for the VYCAP size-based filtration procedure (figure 13). When using paraformaldehyde (PFA), cells were fixated by the formation of formaldehyde which covalently bonds to the molecules of the cells. The cells were then permeabilized with saponin, which is part of the antibody mix (protocol 6). The FIX&PERM is a ready-made kit, where fixation and permeabilization happens separately by a component A (fixation) followed by a component B (permeabilization).

Six blood samples were collected from healthy volunteers directly in TransFix blood collection tubes, and four of these samples were spiked with 1000 ZR-75-1 cells counted using protocol 4. Two samples were reserved as negative controls. Following a 24-hour incubation, all the samples were analysed following protocol 6 for VYCAP size-based enrichment. At the fixation step (see protocol 6 step 12), three samples were fixated with 4% paraformaldehyde, followed by the permeabilization by saponin. Three samples fixated and permeabilized with the FIX&PERM kit which is recommended by VYCAP. Analysing the samples under fluorescence microscopy, we observed an average recovery rate of 58,9% for fixation with paraformaldehyde, and 52,4% for fixation/permeabilization with FIX&PERM. The difference was, however, not significant, and determining the most optimal reagents was difficult. Figure 14 gives an overview of these findings. A detailed overview over optimization of permeabilization and fixation is presented in appendix B4.

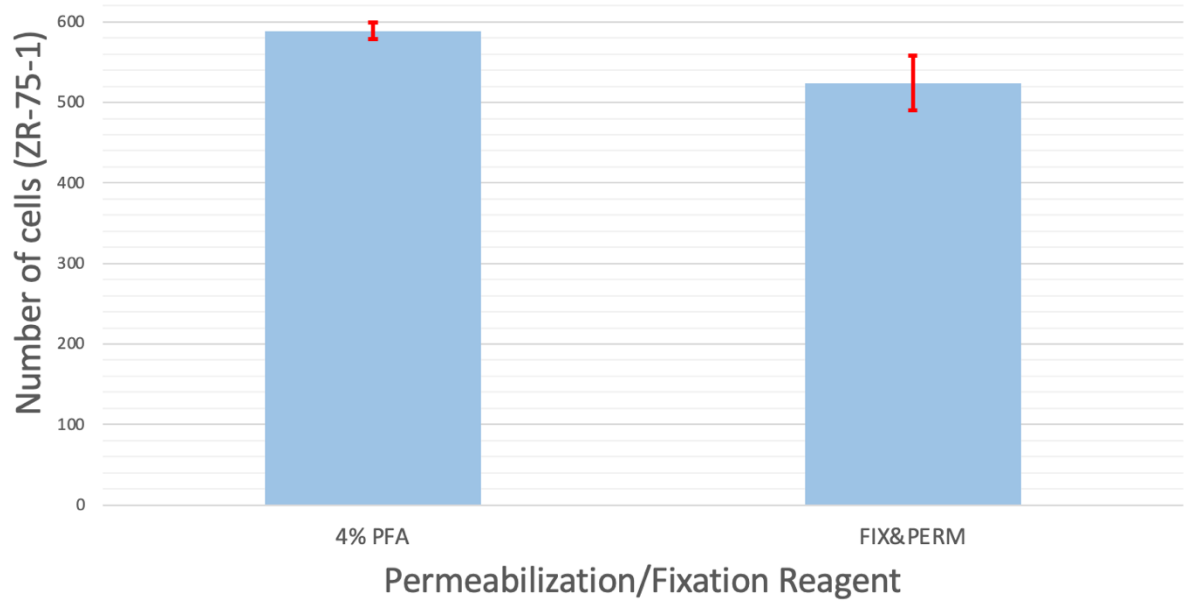

Figure 14: Barplot illustrating recovered cells in optimization of permeabilization and fixation reagent*. Two permeabilization reagents 4% paraformaldehyde and FIX&PERM were tested. All blood samples were derived from healthy individuals in TransFix blood collection tubes spiked with 1000 ZR-75-1 cell line cells. The barplot displays total number of recovered cells (blue). Error bars show the standard deviations. PFA = paraformaldehyde*

Under the microscope, samples that were fixated with 4% paraformaldehyde showed intact, well-shaped cancer cells with an even and bright stain. The blood cells were well organized in the microwells of the filter. None of the cancer cells showed signs of CD45-staining. This is visualized in figure 15 below.

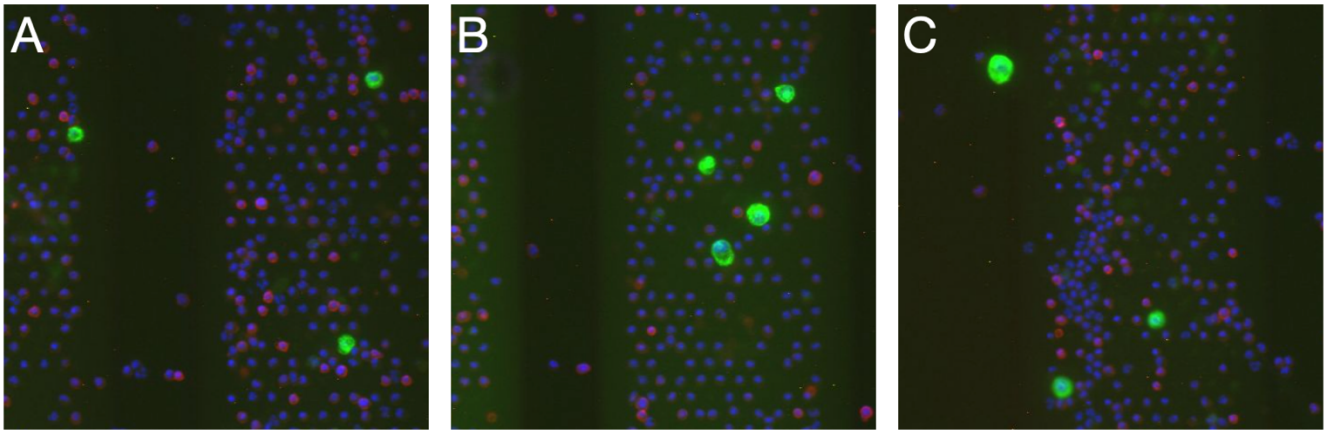

Figure 15: Immune fluorescence microscopy analysis of enriched fraction using PFA fixation. *The blood collection tube used in this was TransFix.The cells were stained with anti-keratin antibodies (green), anti-CD45 antibody(red) and DAPI (blue). A, B and C show fractions of cells from spiked-in samples with ZR-75-1 cell line cells after VYCAP size-based filtration. Pictures are taken in the Metafer software using 10x objective.*

The samples fixated and permeabilized with the FIX&PERM kit also show intact, well-shaped cancer cells. Most cancer cells also have an even and bright stain, similar to cells of 4% paraformaldehyde fixation. Generally, the samples of both permeabilization/fixation reagents look relatively similar, with little to no outstanding differences. Figure 16 below illustrates cells from the FIX&PERM permeabilization.

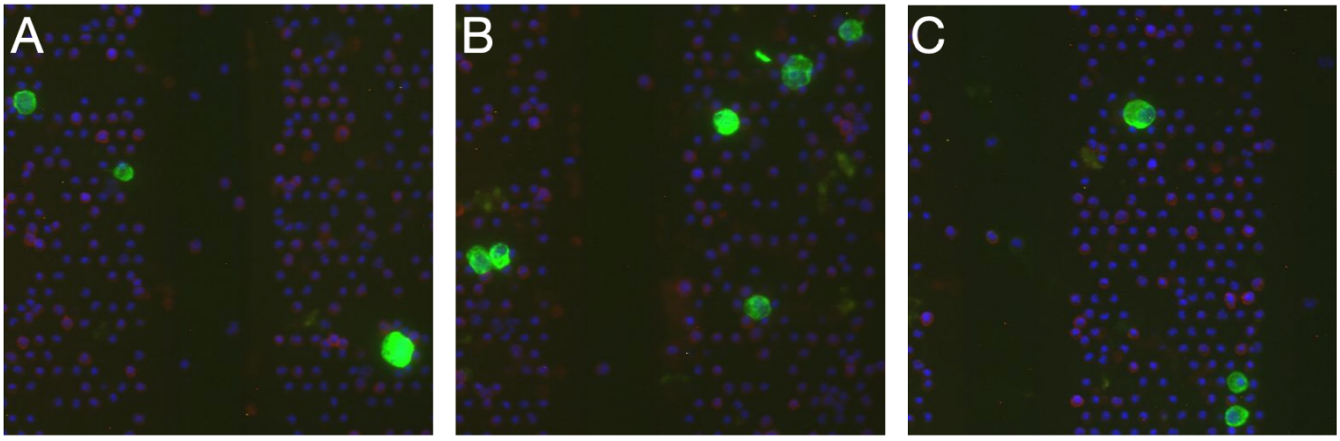

Figure 16: Immune fluorescence microscopy analysis of enriched fraction using FIX&PERM. *The blood collection tube used in this was TransFix.The cells were stained with anti-keratin antibodies (green), anti-CD45 antibody(red) and DAPI (blue). A, B and C show fractions of cells from spiked-in samples with ZR-75-1 cell line cells after VYCAP size-based filtration. Pictures are taken in the Metafer software using 10x objective.*

Comparing figure 15 and 16, both illustrate bright keratin-stained cancer cells, with a large amount of CD45-stained blood cells organized in microwells. Considering the manufacturer's (VYCAP) protocol, FIX&PERM is the recommended permeabilization, in combination with using TransFix blood collection tubes. There is also little

significant difference between samples of PFA-fixation and FIX&PERM in this optimization experiment. Furthermore, applying FIX&PERM permeabilization is simpler and less time consuming than using PFA, and all this considered, it was concluded that the FIX&PERM cell permeabilization kit in combination with TransFix blood collection tubes was the most optimal for the size-based enrichment approach used in project.

3.3 Patient Samples

A total of seven blood samples were analysed, collected from seven individual patients diagnosed with metastatic breast cancer (see section 2.1.2). The samples were analysed according to the VYCAP size-based filtration and immunofluorescent staining (protocol 5). Most patient samples were analysed before the optimization experiments. This means that most patient samples were analysed using CellSave tubes and paraformaldehyde fixation, which was later suggested (section 3.2) to not be the most optimal. After optimization, patient blood samples were collected in TransFix blood collection tubes and subsequently fixated and permeabilized with the FIX&PERM kit.

Results from the analysis of all patient samples are presented in table 10. CTCs were identified following protocol 8. Both clear CTCs and potential CTCs were identified according to their staining pattern and morphology. Out of the seven collected blood samples, only one sample (14%) appeared to contain CTCs. Sample 2 (table 10) contained 2 single CTCs, and additionally 2 CTC clusters consisting of 3 CTCs each. All these cells were keratin-positive, and CD45-negative, however, the keratin-staining was weaker than the staining of the typical cell line cells, and the cells are therefore marked red in table 10 below One of the two CTC clusters found in patient sample 2 is depicted in figure 17 below.

Table 10: *Results of patient sample analyses. Blood samples were collected from seven individual patients prior to receiving cancer treatment. Blood was collected in either CellSave blood collection tubes or TransFix blood collection tubes.*

Sample number	Time from sample to analysis (h)	Blood collection tube	Volume sample analysed	Permeabilization	CTCs	Possible CTCs	CTC-clusters
1	72	CellSave	3,2 ml	4% PFA	0	6	0
2	24	CellSave	9,0 ml	4% PFA	NA	NA	NA
3	24	CellSave	9,0 ml	4% PFA	2	36	2
4	24	CellSave	9,0 ml	4% PFA	0	38	0
5	24	TransFix	9,0 ml	FIX&PERM	0	14	0
6	24	TransFix	9,0 ml	FIX&PERM	0	18	0
7	24	TransFix	9,0 ml	FIX&PERM	0	0	0

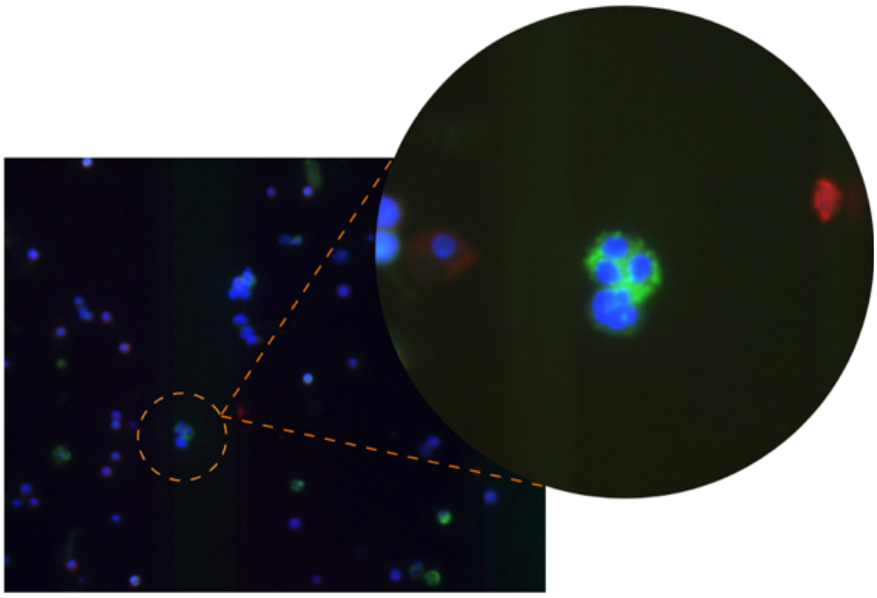

***Figure 17: CTC cluster in patient samples**. The cells were stained with anti-keratin antibodies (green), anti-CD45 antibody(red) and DAPI (blue). Cancer cells are stained green with keratin antibodies, leukocytes are stained red with CD45 antibodies, and cell nuclei are stained blue with DAPI. CTC clusters consist of >1 CTCs that are positive for keratin and negative for CD45. Pictures are taken using the Metafer software with 10x objective (square photo) and the ISIS software with 40x objective (round photo).*

It was also observed cells in all patient samples that appeared to be very similar to the weakly keratin-stained cell line cells from section 3.2.2. The discovery of these cells may propose a problem when analysing patient samples. For examples of these cells in both “filter”-samples and patient samples, see Appendix B6. An overview of CTCs, possible CTCs and CTC clusters may be found in figure 18.

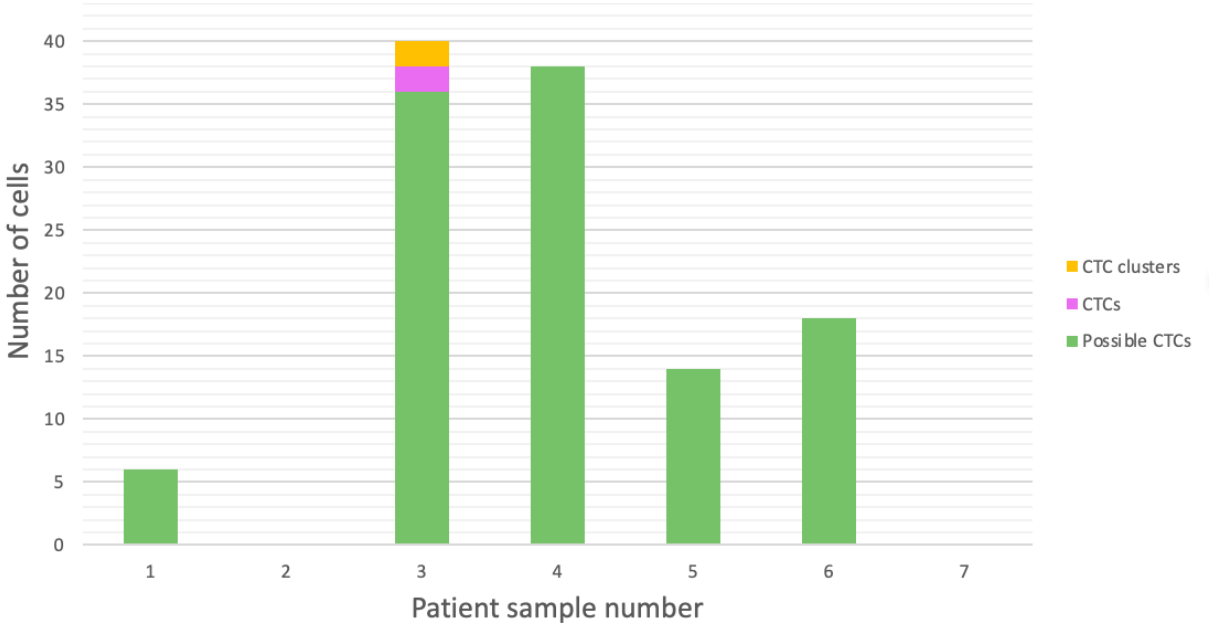

***Figure 18: Barplot over CTCs, possible CTCs and CTC clusters patient samples 1-5.** The types of cells found in each of the samples 1-5 are presented; possible CTCs (green), CTCs (pink) and CTC clusters (yellow). All blood samples were derived from patients with metastatic breast cancer before receiving cancer treatment.*

4. Discussion

4.1 CTC Enrichment and Detection

4.1.1. Circulating Tumour Cell Detection in General

Circulating tumour cells are a huge area of interest, used in numerous clinical trials as interesting biomarkers for cancer. Although CTC detection has shown promise as a non-invasive diagnostic tool for cancer, there are still several challenges associated with it. One of the primary challenges of CTC enrichment and detection is the low concentration of CTCs in the peripheral blood. For most patients, a 10 ml blood sample contains a number of CTCs ranging from 1-10 cancer cells. This makes their isolation and detection a significant technical and analytical challenge [36]. Moreover, CTCs can appear in various shapes, sizes and have different surface markers. The heterogeneity of CTCs makes it challenging to develop standardized approaches for their detection and enrichment [31].

As proposed in chapter 1.2.3, the most widely used methods for CTC enrichment are the marker-dependent techniques, with the epithelial cell adhesion molecule (EpCAM) being the most widely used cell surface protein marker for positive selection of CTCs. According to Pantel et.al (2019), positive selection based on marker-dependent techniques might introduce a problem related to CTC heterogeneity. The CTC might not express the specific chosen marker, which might result in the loss of detection of some CTCs [31]. For example, there is a significant challenge linked to CTC detection in blood due to their ability to undergo epithelial-to-mesenchymal transition (EMT). During EMT, epithelial markers such as EpCAM can be down-regulated, which may make it difficult to detect these CTCs using the standard EpCAM-based detection method and may result in a significant underestimation of the total number of CTCs in the circulation [29]. In a study using the CellSearch system, it was found that a basal-like breast cancer cell line with features of mesenchymal phenotype expressed EpCAM levels that were too low to allow capture using such antibodies [45].

Another major challenge of CTC enrichment and detection is their short lifetime and low survival rates in the blood. The mechanisms of CTCs include EMT, intravasation into the blood stream, extravasation into distant tissues and the formation of new metastases. Out of all the cancer cells that enter the circulation, only a limited amount of CTCs are able to extravasate into distant tissues and form distant metastases [29]. Due to shear stress, anoikis and immune surveillance, the average lifetime of a circulating tumour cell is estimated to be around 1-2 hours, compared to

red blood cells average of 120 days and white blood cells varying from days to years [34, 46, 47]. Several studies also show the interference of CTCs with other blood cells such as platelets, neutrophils and CAFs, which may protect the CTCs from being discovered with current CTC detection methods, in addition to help facilitate their extravasation and metastatic abilities [34]. All these challenges can result in poor reproducibility of CTC detection, which ultimately makes it challenging to accurately quantify CTCs and utilize them in the clinic.

4.1.2 Challenges and Advantages of Size-based Enrichment of CTCs

During this thesis, CTC enrichment was achieved through size-based approaches, which are techniques that capture CTCs based on their larger size, compared to most leukocytes. Normal blood cells may have a variety of sizes ranging from 5 µm for the smallest leukocytes, and up to 20 µm for larger leukocytes. CTCs may have diameters ranging from 6-20 µm [31], yet it is generally believed that CTCs are larger than the average leukocyte. Various size-based techniques are available, including microfiltration methods that utilize the passing of blood through size-calibrated micropores [31]. Blood is passed through pores which trap the large CTCs and lets the small blood cells pass. One prominent technique is the Parsortix system, which is a microfluidic technology for separating cells by capturing rare cells based on cell size and deformability. According to Parsortix, the epitope-independent process enables the capture of a variety of rare cell types, including both epithelial and mesenchymal cancer cells that marker-dependent techniques like the EpCAM-based techniques might not [48]. Other size-based systems include the RareCyte, iSET, Vortex and VYCAP systems. The RareCyte CTC detection system isolates CTCs based on density and has shown promising results of detecting CTCs in blood samples from patients with metastatic breast cancer [49]. One study showed that out of 100 samples obtained from patients with progressive metastatic breast cancer, 75% of samples had at least one detectable CTC in 7.5ml of blood, compared to 65% of the samples analysed with CellSearch [49]. The iSET and Vortex systems are other marker-independent systems used to trap, isolate and identify large CTCs [50, 51], similarly to the VYCAP system, which is used during this thesis and discussed further in 4.2.2.

Many studies argue that marker-dependent enrichment approaches, like the CellSearch system, are suboptimal due to the CTC heterogeneity and loss of epithelial antigens during EMT. Panabieres et al. (2014) suggests that the perfect CTC marker is one that is expressed on all CTCs, and at the same time not on other hematopoietic cells, and one that is never repressed during invasion and in the circulation [36]. To our knowledge, one such marker does not exist – there is no universal marker that is expressed on all CTCs [52]. For this reason, many studies

suggest marker-independent approaches like size-based filtration to be preferable for capturing a wide variety of CTCs, independent of their surface markers. In addition, there has been reported EpCAM-positive cells in the circulation of patients with benign colon disease, which may rise the possibility of false positive findings [52]. In a study by de Wit et al. (2014), they tried to develop a method for establishing whether CTCs escaped the EpCAM-dependent enrichment of CellSearch by collecting the blood discarded by the CellSearch system after immunomagnetic selection. This blood was then analysed by a size-based microfiltration method, followed by immunofluorescent staining and microscopy. The sample did show keratin-stained cells after size-based filtration that was suggested to be CTCs [53]. The combination of marker-dependent and size-based CTC enrichment might be a good solution to minimizing the problems that inevitably occur on either side of the spectrum.

While size-based enrichment methods have shown to be a successful tool in capturing CTCs, there are still several challenges associated with these methods, similar to those with marker-dependent methods. Although a successful technique in many studies, there is a high probability of losing small CTCs that may be present in the sample. Some CTCs will inevitably be smaller than the micropore size of the microfiltration devices used, and these cells may be filtered away and lost [29]. On the other hand, many studies imply that size-based filtration is better for capturing a variety of rare cells compared to marker-dependent techniques, including EMT cancer cells, as conventional marker-dependent enrichment methods may not capture a large number of CTCs due to their loss of epithelial antigens [54].

Despite the inherent challenges associated with size-based enrichment methods for CTCs, these techniques do have several advantages alongside the ability to capture EMT CTCs. The method is highly efficient, as filtration enables the rapid enrichment of CTCs from a large volume of sample in a short period of time. Moreover, this technique involves minimal sample processing, which is advantageous in preserving the integrity of the sample and minimizing false negatives [52]. The procedure is relatively easy to follow and allows for the analysis of multiple samples in a single day. Combining size-based filtration with immunofluorescent staining enables the identification of CTCs with high sensitivity and specificity, enhancing the accuracy and reliability of the CTC detection.

4.1.3 Clinical Relevance

The EpCAM-based CellSearch system is the only clinically approved system for detection of CTCs. The system is cleared by the US Food and Drug Administration (FDA) and is the only system used for detection of CTCs in metastatic breast,

prostate and colorectal cancer patients in the clinic. The detection of CTCs in the peripheral blood of cancer patients using the CellSearch system is associated with a decreased progression-free survival, and a decreased overall survival of the patients [37]. The CellSearch system is also used to evaluate the presence of CTCs at any time during the disease, which allows for the assessment of patient prognosis.

Despite the EpCAM-based CellSearh method being the standard method for CTC detection in the clinic, many researchers believe that introducing marker-independent techniques like size-based filtration will improve the isolation and detection of CTCs in cancer patients due to their ability to capture CTCs with down-regulated expression of EpCAM markers. In a study by Poruk et al. (2016), they assessed CTCs with epithelial and mesenchymal phenotypes as potential prognostic biomarkers for cancers using a size-based filtration method. They found that the presence of epithelial CTCs was associated with a poorer survival, and that the presence of CTCs expressing both epithelial and mesenchymal markers was associated with cancer recurrence [55]. This study was done on patients with pancreatic adenocarcinoma, and it was concluded that the enumeration of CTCs using size-based methods do provide prognostic significance for patients with this type of cancer.

In spite of this, not enough studies are based on the clinical relevance of the size-based approach to CTC detection, and standards for evaluating the technical and clinical performance of marker-independent methods are still lacking. Apart from the CellSearch system, almost all the available up-and-coming CTC-technologies lack large multicentre trials to show clinical validity. In other words, there is a lack of evidence that marker-independent is actually clinically relevant [56]. There is still extensive research being done on the marker-independent techniques, yet it is time consuming and expensive, and it is a challenging task to ensure the technical validity of these approaches. CellSearch remains the gold standard method for CTC detection in clinical context, and the detection of CTCs using marker-dependent techniques are often complemented by additional genomic analyses to avoid false positives. In addition, the use of antibody combinations targeting various markers that cover the heterogeneity of CTCs is utilized to avoid false-negatives [36].

4.2 VYCAP Size-based Enrichment and Detection of CTCs

The average recovery rates from the validation experiments in this project were found to be 16% for MCF-7 and 27% for ZR-75-1 cell lines, which are quite low numbers compared to reported recovery rates for cell line cells using size-based filtration techniques. The VYCAP system, which combines size-based filtration with

imaging, reportedly achieves recovery rates of 65-79% for epithelial and mesenchymal human cancer cell lines [57]. In a study by Pillai et al. (2017) an average recovery rate of 87% was proposed for the SKBR3 cell line using a size-based microfiltration approach [54]. Similar to this, Vasantharajan et al. (2022) assessed a size-based CTC enrichment method for detecting CTCs in colorectal cancer using healthy blood samples spiked with cell line cells and showed recovery rates of >85% for the cell line cells [58]. In a previous experiment using the same VYCAP protocol and the ZR-75-1 cell line, an average recovery rate of 48,7% was observed [43], which is still considered low but significantly higher than the results of this thesis.

There are several reasons that may explain low recovery rates. The size of the cell line cells compared to the micropore size may contribute a problem as some cells may have been smaller than the pore size of the microsieve. VYCAP has developed a size-based enrichment method for CTCs in which the CTCs are separated from blood cells based on their size. The filter used in the VYCAP protocol contains around 160 000 precise pores of 5 ± 0.2 µm, and the silicon nitride filter membrane of the VYCAP microsieve is 1 µm thick. The filter membrane requires a low pressure compared to other size-based filtrating systems, resulting in less damage of the captured cells, according to VYCAP [59]. The optimal pore size for size-based enrichment of CTCs may vary depending on the size of the CTC of interest, as well as the type of microfiltration device used. VYCAP states that even though the difference between the size of the CTCs and other blood cells is little, the 5 µm pores of the device will allow for the precise capture of CTCs [59]. This statement does not seem to correspond very well with the results from the validation experiment of this thesis. One can question whether some cell line cells are better for filtration than others, and the low average recovery rates from this experiment, even after switching to manual cell counting, further supports this statement.

There were several problems with cell line spiking and cell counting, which may have contributed to the low recovery rates (see Appendix B1), and there was suspected to be a problem with imprecise spiking. The Countess automated cell counter was used for these first experiments (see protocol 3). According to the manual, the device is able to count cell concentrations ranging from 10^4-10^7 cells/ml [60]. One suspected reason for low recovery rates was that the cell concentration of the cell suspension used was too low for the Countess to be able to accurately count. In an experiment done by comparing the Countess automated counter with manual cell counting, there were quite significant differences in cell concentration results within the same cell suspension. Even by counting the same sample multiple times with Countess, different results were provided each time. For this reason, it was decided to switch to manual cell counting (see protocol 4), which gave the results of 99,6% and 96,0% recovery for cell line cells without filtration. However, even after switching to manual

cell counting, recovery rates still remained fairly low for spiked-in blood samples, indicating that many cells may be lost during handling or filtration, and not during cell counting.

During validation experiments, CellSave blood collection tubes were used in combination with 1% paraformaldehyde fixation. This is in opposition to the VYCAP protocol in which TransFix tubes and FIX&PERM is used, which may have contributed to the lower recovery rates. It is possible that the pressure applied when performing validation experiments lead to the destruction of tumour cells due to the combination of too weak fixation from the CellSave tube, and too high pressure from the VYCAP pump. By looking at the results from the optimization experiments, it was determined that the TransFix tube combined with FIX&PERM was the most optimal combination. Had these factors been implemented from the very start of validation experiments in addition to manual cell counting as opposed to automated cell counting, maybe results would have been different and more satisfactory. On the other hand, manufacturers did ensure the CellSave tubes as perfectly fine to use for this experiment, which again only raises more questions. Other reasons for low recovery rates include possible clogging of the filtration membrane, loss of cells during the process, or general operator error. It is possible for the filtration membrane to become clogged with debris, as was observed during optimization of blood collection. Using the 1% paraformaldehyde tubes, the microsieve filter was clogged, and the sample could not be analysed. CTCs may also be lost during the process due to pipetting, filtration, and washing steps. Finally, operational errors, particularly during the cell counting step, may have contributed to the low recovery rates observed in this project. The immunofluorescent staining step was ruled out as being a source of low recovery rates, as a result of the experiment of pipetting ZR-75-1 cell line cells directly on to the microsieve filter without filtration. A total of 1000 cells were applied to the filter, and almost all cells were recovered using immunofluorescence microscopy, as results gave the average recovery of 97,8%.

4.3 Optimization of Protocols

The choice of blood collection tubes often remains an underrated variable of the pre-analytical phase of blood sample analysis [61]. The components of the blood collection tubes can affect the specimens they carry, and may interfere with laboratory results [62]. During most of this project, CellSave blood collection tubes were used. Initially, the experiment was intended to be based on EDTA blood collection tubes, which do not contain fixatives. However, previous experiments using the same VYCAP protocol, and the same cell line showed that the cells of the EDTA tubes appeared to be destroyed by the pressure of the filtration [43]. It was therefore decided to switch to using a fixative, and the choice fell on CellSave tubes due to

them being readily available at the lab. CellSave tubes are optimized for stabilizing circulating tumour cells and improving the reproducibility and reliability of the CTC analysis [63]. They allow for the stabilization of CTCs for up to 96 hours, yet no lower limit is implied [63]. In the beginning of validation experiments, spiked-in blood samples were incubated for both 1 and 24 hours with cell line cells before analysis, and results indicated more positive results for a 24-hour incubation as the cells appeared more intact. In addition, previous experiments using the same protocol established a 24-hour incubation time as optimal, and for this reason, 24 hours were the standard incubation time used in further spiking experiments.

As mentioned, VYCAP recommends the TransFix tube, which is designed to stabilize CTCs for up to 5 days. Results of optimization experiments point to these tubes being a better fit for the detection of circulating tumour cells as these samples have a better overall recovery and the overall impression of more intact cells (section 3.2.1). This corresponds to a study done by Koch et al. (2020), where a comparison of CellSave, TransFix, Streck and EDTA blood collection tubes were carried out using a marker-independent enrichment approach, both in spiking experiments and on patient samples [64]. The study reports that in cell line spiking experiments, the mean recovery was highest for TransFix tubes at 64,0% recovery, compared to CellSave tubes that only showed a recovery of 16,7%. When applying the different blood collection tube fixatives to patient samples, the highest rates of finding a CTC-positive sample (≥ 1 CTC) were reached for TransFix and Streck tubes (46,2%). In comparison, only 30,8% of samples using CellSave tubes were CTC-positive [64]. The study concludes on TransFix being the most optimal tube, as is concluded in this thesis. We generally observed more cells in the samples collected in TransFix tubes, indicating that the cell fixative in TransFix makes cells more rigid and less likely to be damaged by the pressure of filtration. This could on the other hand propose a problem of filter clogging and less efficient enrichment. More cells may also lower the specificity of the sample, which may elevate the chance of false positive findings and can lead to unnecessary diagnostic tests or treatments. When analysing the blood collection tubes containing 1% paraformaldehyde, the sample could not be filtered through the microsieve due to a coagulation that had clogged the filter. Most likely, the fixation of the paraformaldehyde had been too strong, and the samples were therefore not analysed. The cells of the Streck tubes appeared destroyed and less intact than the cells of the other tubes, and no more attention was therefore given to this tube.

When comparing permeabilizations, there was not observed a very significant difference between the 1% paraformaldehyde permeabilization versus the FIX&PERM cell permeabilization kit. Permeabilization of the cells allow for larger molecules like antibodies to enter the cells and is an essential step with regard to immunofluorescent staining. At the point of permeabilization, cells are already

fixated, and the permeabilization step may not affect the cells to the same extent. Due to FIX&PERM being recommended by VYCAP, and that the protocol for using this permeabilization set is easier and less time consuming, it was declared as optimal for this project.

4.4 Immunofluorescent Staining and Microscopy

Immunofluorescent staining with fluorescently labelled antibodies was used to distinguish between tumour cells and blood cells of the analysed samples. There was observed a problem during analyses of this thesis where some cells of the sample were positive for both keratin and CD45. This was observed to be the case for both spiked-in samples, and for negative control samples. In experiments of cell line cells without filtration, there was no CD45 signal from any cells. The cell line cells are however only used as a representative for breast cancer cells, and there are actually instances of breast cancer cells expressing CD45, even if the occurrence is low [65]. Some of these keratin-positive, CD45-positive cells were distinctly polymorphonuclear, resembling the nucleus of white blood cells like neutrophiles, eosinophiles or basophiles. These cells were easily distinguished as leukocytes and were therefore not counted as CTCs or possible CTCs. Still, some of these cells did not appear as polymorphonuclear, and these were present in both negative control samples and in patient samples. In patient samples, these cells were counted as “possible CTCs” (see table 10). There is a possibility of epithelial-specific antibodies like cytokeratins to bind to non-tumour, non-epithelial cells through non-specific labelling [66]. In an article by Paterlini-Brechot and Benali, they state that the percentage of cytokeratin-positive cells in normal controls may range from 0-20%. There are reports of antibodies against keratin (in addition to other epithelial-specific antigens) to bind both specifically and non-specifically to macrophages, plasma cells and other nucleated hematopoietic cell precursors [66]. Often, these cells can be morphologically difficult to distinguish from CTCs, and large efforts were put into the analysis of patient samples due to this occurence. In conclusion, the cells detected as “possible CTCs” during this project were generally believed to not be CTCs. All these cells were small in size, and generally had a small CTCs nuclear to cytoplasmic ratio compared to the cancer cell line cells. In addition to this, there were also problems with staining of the microsieve filters, which contributed to the already difficult task of counting CTCs in patient samples.

In all experiments of cell line cells without filtration, there was observed a varying degree of keratin-staining of the cells. Most cells had even, bright keratin staining, however some cells only appeared to have very little to no stain (see appendix B5 for photo examples). This proposes the question of whether all CTCs can be detected using the antibody mixture in protocol 5. The cytokeratin AE1/AE3 antibody binds to keratins 1-8, 10, 14-16 and 19 [67], whereas the cytokeratin C-11 antibody binds to

keratins 4, 5, 6, 10, 13 and 18 [68]. In an article by Polioudaki et al. (2015) it is reported that keratins are differentially expressed among different breast cancer cell lines, and that the expression is down-regulated during metastatic spread. It has also been suggested that a modulation or down-regulation of keratins due to EMT frequently occurs in CTCs of breast cancer [69]. However, there is little research on the varying expression of keratins in the same cell line. Still, this proposes the suggestion of using an antibody cocktail of different cytokeratins targeting different keratins when analysing patient samples. In the VYCAP protocol, they use the CD16 antibody, which binds to the surface of hematopoietic cells like for instance NK-cells, activated monocytes, macrophages, and neutrophils. It is used for negative selection of CTCs, as CTCs should be classified as being CD16 negative [59]. It could be interesting to test using this antibody to determine if it has an impact on either false negative or false positive cells in the samples.

Furthermore, some manufacturing defects in the VYCAP equipment used in the staining protocols and during fluorescence microscopy interfered with certain analyses. For instance, in patient sample 2, a defect in the VYCAP filtration unit became apparent only after the sample had been added, resulting in the loss of the sample. Moreover, the VYCAP microsieve filter was sometimes produced in a manner that caused the cover glass to not lie flat on top of the micropores, thereby preventing successful scanning of the sample in Metafer. Additionally, using the 40x objective to obtain images of the samples posed a challenge, particularly when the target of interest was located in the outer parameters of the filter sample, as the objective collided with the plastic edges of the microsieve. The problem did not occur when using the 10x objective. When conferring with a VYCAP employee, it was apparent that they were aware of the problem, and that they had adjusted their microscope slide adapters to avoid the problem. However, even after receiving the new slide adapters, the problem was not solved. Hopefully there is a solution to this problem in the future.

4.5 CTC Detection in Patient Samples

Only one out of seven (14%) patient samples had detectable CTCs and CTC clusters, classified as being keratin positive, CD45 negative and having a slightly larger size. Even so, these cells still have a relatively weak keratin stain, and look relatively different to the staining of most of the cell line cells. 6 out of 7 patient samples had cells classified as “potential CTCs”, which were keratin positive, CD45 positive and were observably mononuclear. The number of potential CTCs in the samples ranged from 6 at lowest, to 38 at highest. In an article by Andree et al. (2019), they applied the VYCAP size-based enrichment method to patients with metastatic breast and prostate cancer and compared the results with the results from

the CellSearch system [70]. In clinical samples, they actually found that the VYCAP system only captured 0-5% of CTC detected by the CellSearch system in a parallel experiment. They also raised the question of where the cells disappear and argued that even if the protocol works well with spiking experiments, cell line cells have a known size. The actual size of the CTC in individual patients is unknown and may be very variable. A potential solution could be to use smaller micropore sizes. The VYCAP micropore has a size of 5 µm, and decreasing the size would maybe enable the capture of smaller CTCs, yet it would be important to calibrate the size to allow for the flow of leukocytes, and to not block the pores.

There are also studies that show size-based CTC enrichment techniques to be better at capturing CTCs compared to marker-dependent techniques. In a study by Huebner et al. 2018, they compared the CTC enumeration of the CellSearch system to a filtration-based method [71]. They analysed 60 blood samples obtained from patients with metastatic breast cancer and found that the CTC positivity rates was 56,7% for CellSearch, and 66,7% for the filtration-based method, and concluded that the method was suitable for determining CTC counts in metastatic breast cancer patients.

Only three blood samples were analysed under the established optimal conditions with TransFix blood collection tube and FIX&PERM reagent, and it would have been interesting to see the results from more patient samples with these requirements. CTCs are considered an extremely rare cell, and most cancer patients only have a concentration of 1-10 cells per 10 ml of blood [36]. Approximately, 1 CTC per ml of blood will be surrounded by around $5*10^6$ white blood cells and $5*10^9$ red blood cells, which proposes a huge technical difficulty in the CTC enrichment of patient samples [53], as was discovered during this project.

4.6 Further Research

With regards to this thesis, an optimized method for the recovery of cancer cell line cells was established, yet a lot more samples need to be analysed to determine its clinical relevance. Preferably, more validation experiments should be done using the TransFix blood collection tube and FIX&PERM after determining them as optimal. Further optimization of the method with regards to especially pressure and filtration time should also be implied. All samples in this experiment were filtered based on the statement from VYCAP that reads “The pressure setting is correct if the blood passes the filter with a flow rate of approximately 1-3 ml blood/minute” [44]. The VYCAP protocol states to set the pressure to 200 mbar. For validation experiments using CellSave blood, pressures needed to be way lower, around 50-80 mbar, to obtain the ideal speed. At higher pressures, cells appeared to be less intact and more smudged

out against the background of the microsieves. During experiments with TransFix tubes, it was observed that pressure needed to be a little higher to obtain ideal filtration speed, however this should be optimized further with more spiked in samples.

Future experiments should also focus on the interaction of the CTCs with other blood cells. During this thesis, very few CTCs were found, and investigating their blood cell interactions was therefore a huge challenge. Determining CTCs interaction with especially platelets and neutrophils, but also other blood cells like erythrocytes, macrophages, and natural killer cells (NK-cells), can contribute to better understanding the mechanisms behind the CTCs. Focusing on the underlying mechanism in the bonds and interactions between these cells may help understanding the nature of the CTCs at a deeper level, as interactions with other blood cells may contribute to CTC survival in the blood, and accordingly propose a potential clinical relevance [34]. It would also be interesting to directly compare the VYCAP method with the gold standard CellSearch. Comparing results from both these techniques would maybe give some interesting knowledge on the size-based method and help in determining its clinical validity.

Size-based enrichment of CTCs has been identified as a promising approach for clinical analysis of cancer patients. While many researchers prefer marker-independent methods for detecting CTCs, there is still a long way to go in determining the clinical validity of such methods. Future research is required to determine the clinical relevance of size-based enrichment, which is a time-consuming and costly procedure. Nevertheless, applying CTC analysis to patient diagnosis and treatment remains an essential aspect of CTC research, as does investigating the correlation between CTC presence and prognostic value. Previous studies have demonstrated that the presence of CTCs in the circulation is correlated with a poorer prognosis [36]. Therefore, additional research on CTCs is necessary to provide an improved prediction of a patient's future health outcomes. There is a huge variety of size-based (and other marker-independent) approaches available, and determining the most optimal technique for CTC detection poses a significant challenge. Consequently, effort should be applied to this area to establish whether marker-dependent techniques are superior for CTC detection or whether a combination of size-based and marker-dependent methods would be the most effective approach. Being able to apply CTC analysis to determining the specific cancer treatment for a specific patient would ultimately be a goal in the understanding of the circulating tumour cell mechanisms and would be both an important and interesting part of the CTC research.

5. Conclusion

We have established an optimized method for size-based enrichment of circulating tumour cells using the VYCAP size-based filtration system coupled with immunofluorescent staining. It was concluded that the TransFix blood collection tube in combination with the FIX&PERM permeabilization kit was the most optimal for this approach. One out of seven (14%) analysed patient samples contained CTCs, and further research should include more patent samples to determine the clinical relevance of the approach.

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7. Appendix

Appendix A – Materials: Filter sets and fluorophores used in immunofluorescence microscopy.

Table 11: *Filter configurations for the Leica microscope. The table provides the desired target, antibody used, filter set used and excitations and emissions in the different filter sets of the microscope.*

Target	Antibody	Filter set	Excitation	Emisson
Leukocyte membrane	Anti-Hu CD45 antibody APC	Filter set 50 (APC)	640/30	690/50
Cancer cell membrane	Anti-Pan Cytokeratin AE1/AE3 eFluor 570 Pan Cytokeratin Monoclonal antibody (C-11) PE	SpGold	547/12	572/22
Cell nuclei	DAPI	DAPI	360/40	460/50
Platelets	Anti-Hu CD61	SpGreen	494/26	536/30

Appendix B1 – Results: Detailed overview of validation experiments.

Table 12: *A detailed overview over validation experiments. Date of analysis, incubation times, filtration pressures and filtration times are provided. Recovery percentage is calculated for counted samples. BCT = Blood collection tube.*

Sample name	Cell line	Date of analysis	BCT	Incubation time (h)	Filtration pressure (mbar)	Filtration time (min)	Number of cells spiked	Number of cells recovered	Recovery percentage
Validation 1	MCF-7	26.01.2023	CellSave	24	75	9	200	34	17,0 %
Validation 2	MCF-7	26.01.2023	CellSave	24	75	9	200	13	6,5 %
Validation 3	MCF-7	01.02.2023	CellSave	1	75	9	1000	200	20,0 %
Validation 4	MCF-7	01.02.2023	CellSave	1	75	9	1000	186	18,6 %
Negative control 1	-	01.02.2023	CellSave	1	75	9	-	-	-
Cell line filter 1	MCF-7	01.02.2023	CellSave	-			1000	540	54 %
Validation 5	ZR75-1	07.02.2023	CellSave	1	75	4	1000	not counted	-
Validation 6	ZR75-1	07.02.2023	CellSave	1	75	4	1000	not counted	-
Negative control 2	-	07.02.2023	CellSave	-	75	5	-		-
Cell line filter 2	ZR75-1	07.02.2023	CellSave	1			1000	not counted	-
Validation 7	ZR75-1	21.02.2023	CellSave	24	75	10	1000	292	29,2 %
Validation 8	ZR75-1	21.02.2023	CellSave	24	75	10	1000	not counted	not countet
Negative control 3	-	21.02.2023	CellSave	24	75	10	-	-	-
Cell line filter 3	ZR75-1	21.02.2023	CellSave	-			1000	996	99,6 %
Validation 9	ZR75-1	10.03.2023	CellSave	24	55	15	1000	162	16,2 %
Validation 10	ZR75-1	10.03.2023	CellSave	24	55	15	1000	304	30,4 %
Negative control 4	-	10.03.2023	CellSave	24	55	15	-	-	-
Cell line filter 4	ZR75-1	10.03.2023	CellSave	-			1000	960	96,0 %

Appendix B2 – Results: Keratin-stained cells in negative control samples

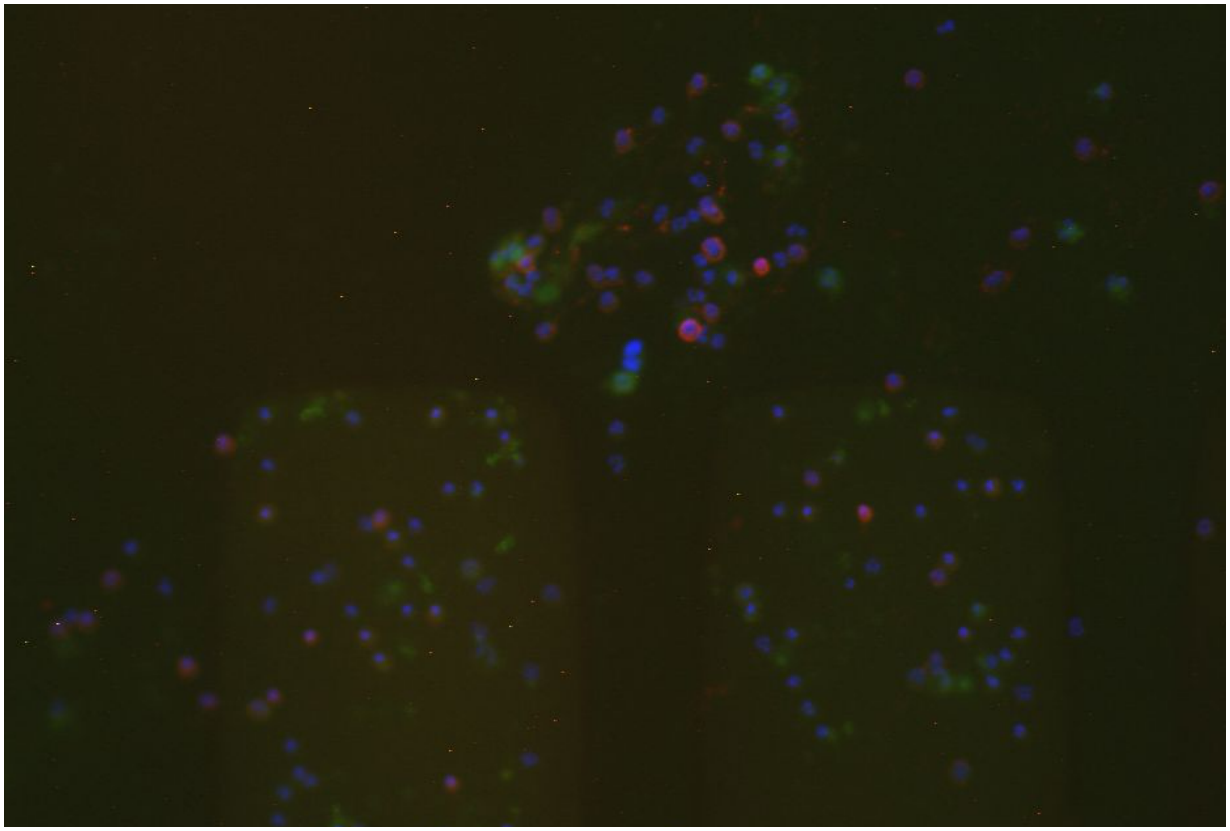

Figure 19: Keratin-stained cells in negative control samples. *Leukocytes are stained red with anti-CD45 antibody, and cell nuclei are stained blue with DAPI. Some cells appear to be keratin-stained despite of this being a negative control sample from a healthy individual.*

Appendix B3 – Results: Detailed overview over optimization experiments of blood collection tubes

Table 13*: A detailed overview over optimization of blood collection tube. Cell line, incubation time, filtration pressure and filtration time are provided. Recovery percentage and average recovery percentage is calculated for all blood collection tubes. NA = Not analysed.*

BCT	Parallel	Cell line	Incubation time (h)	Filtration pressure (mbar)	Filtration time (min)	Number of spiked cells	Number of recovered cells	Recovery percentage
CellSave	1	ZR-75-1	24	50	15	1000	162	16,2 %
	2	ZR-75-2	24	50	15	1000	304	30,4 %
	N	-	-	50	13	-	-	-
	Average						***233***	***23,3 %***
TransFix	1	ZR-75-1	24	65	16	1000	304	30,4 %
	2	ZR-75-2	24	65	15	1000	300	30,0 %
	N	-	-	65	15	-	-	-
	Average						***302***	***30,2 %***
Streck	1	ZR-75-1	24	35	15	1000	240	24,0 %
	2	ZR-75-2	24	35	13	1000	200	20,0 %
	N	-	-	35	15	-	-	-
	Average						***220***	***22,0 %***
1%PFA	1	ZR-75-1	0,5	-	-	1000	NA	NA
	2	ZR-75-2	0,5	-	-	1000	NA	NA
	N	-	-	-	-	-	-	-
	Average	-	-	-	-	-	-	-

Appendix B4 – Results: Detailed overview over optimization of permeabilization and fixation

Table 14: *A detailed overview over optimization of fixation and permeabilization reagent. Blood collection tube, incubation time, filtration pressure and filtration time are provided. Recovery percentage and average recovery percentage is calculated for parallels. BCT = Blood collection tube.*

Permeabilization	Parallel	BCT	Incubation time (h)	Filtration pressure (mbar)	Filtration time (min)	Number of cells spiked	Number of recovered cells	Recovery percentage
4% PFA	1	TransFix	24	65	15	1000	582	58,2 %
	2	TransFix	24	65	15	1000	596	59,6 %
	N	TransFix	-	65	15	1000	-	-
	Average						***589***	***58,9 %***
FIX&PERM	1	TransFix	24	65	15	1000	548	54,8 %
	2	TransFix	24	65	15	1000	500	50,0 %
	N	TransFix	-	65	15	1000	-	-
	Average						***524***	***52,4%%***

Appendix B5 – Results: Weakly keratin-positive tumour cell line cells

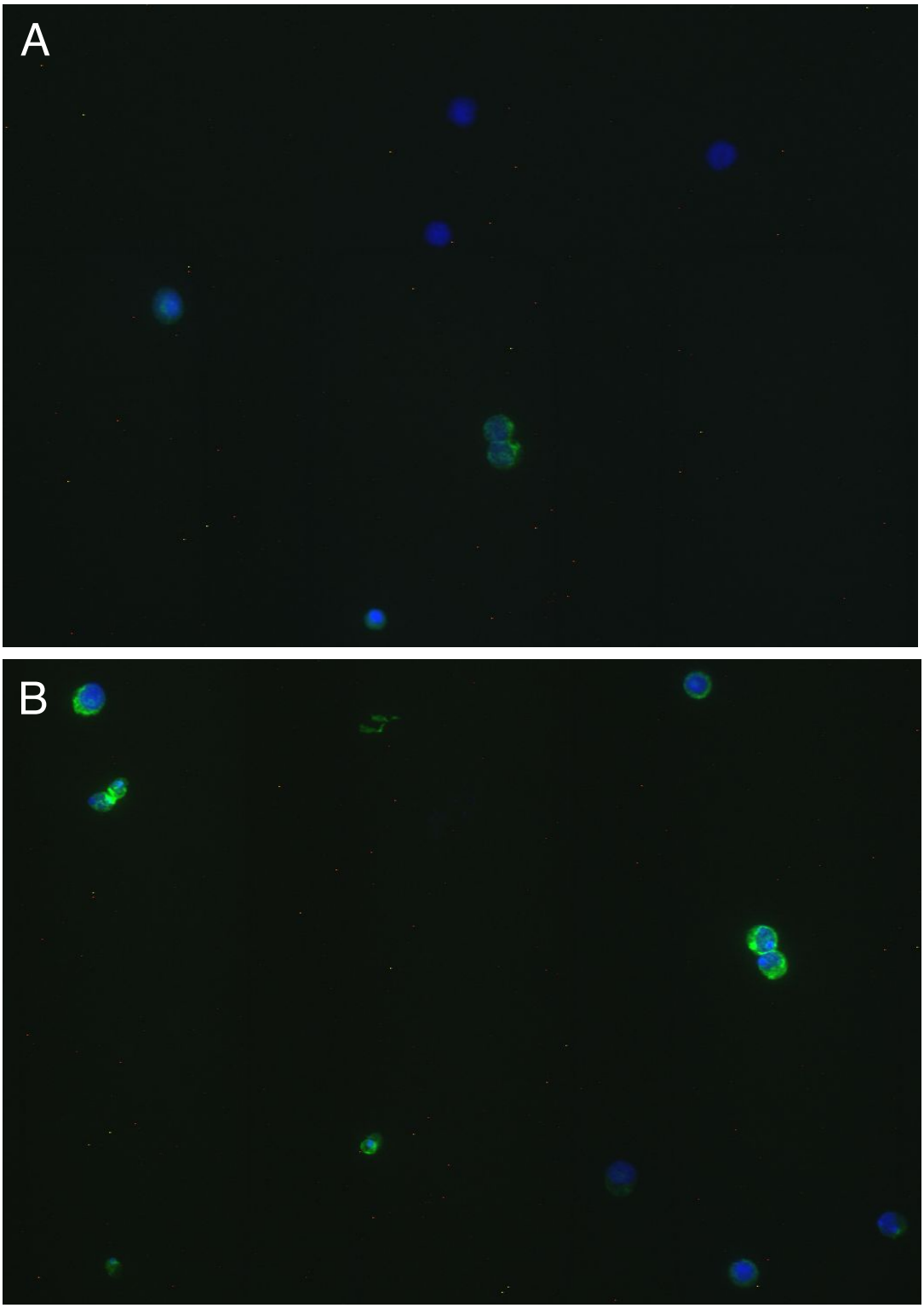

Figure 20: Varying degrees of keratin staining of ZR-75-1 cell line cells. *The ZR-75-1 cell line is stained green with antibodies against keratin, and cell nuclei are stained blue with DAPI. Pictures are taken using Metafer 4 software and the 10x objective.*

Appendix B6 – Results: A comparison of weakly keratin stained ZR-75-1 cells and keratin-negative, CD45-negative cells in patient samples

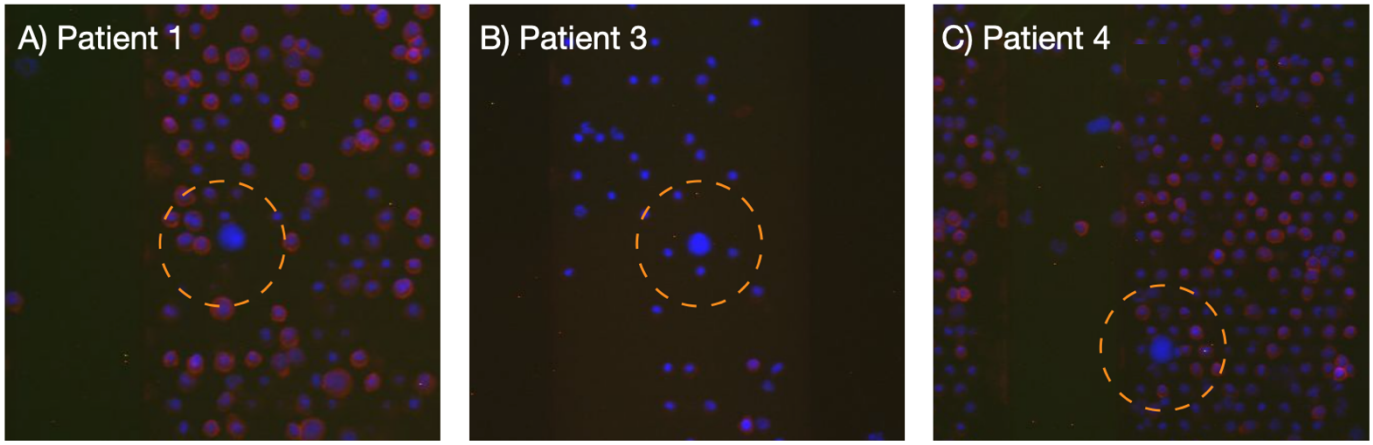

Figure 21: Large keratin-negative, CD45-negative cells detected in patient samples. *Cancer cells are stained green with antibodies against keratin, and nuclei are stained blue with DAPI. Cells of interest are circled in orange. Pictures are taken using Metafer 4 software and the 10x objective. Examples are shown from patient samples 1, 3 and 4.*

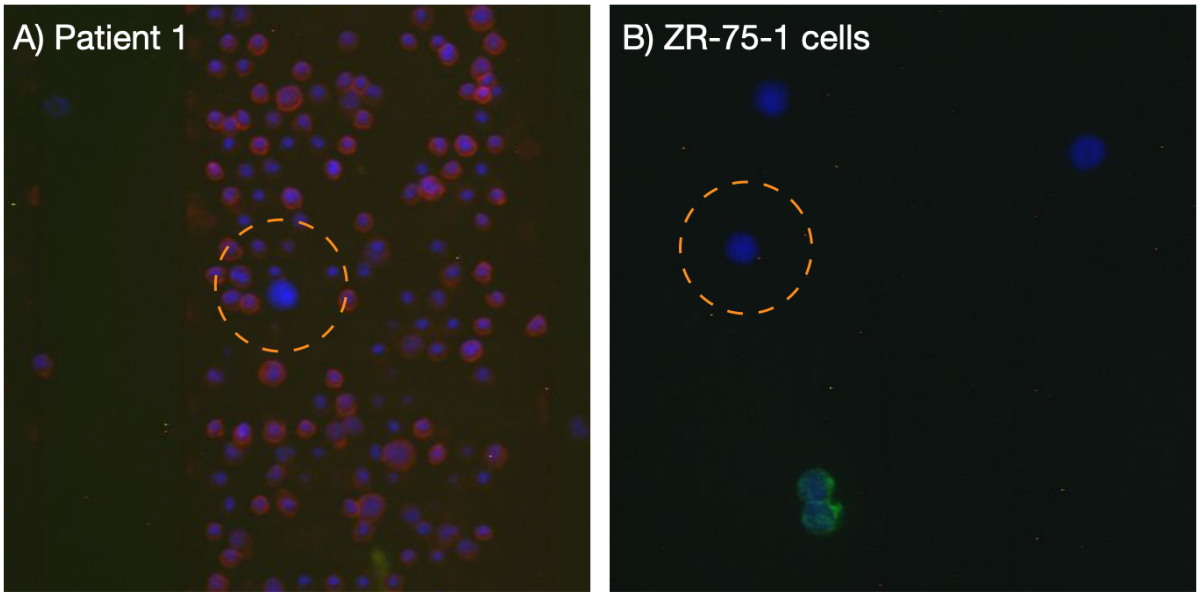

Figure 22: A comparison of large keratin-negative, CD45-negative cells detected in patient samples with weakly keratin-stained ZR-75-1 cell line cells. *Cancer cells and cell line cells are stained green with antibodies against keratin, and nuclei are stained blue with DAPI. Cells of interest are circled in orange. Pictures are taken using Metafer 4 software and the 10x objective. The example shown in A is from patient sample 1.*