FACULTY OF SCIENCE AND TECHNOLOGY

BACHELOR THESIS

Study programme / specialisation:

Biological Chemistry

The spring semester, 2022

Confidential

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Thesis title: Platelet Interaction with Circulating Tumour Cells Isolated by Size-based Filtration from Patients with Metastatic Breast Cancer

Credits (ECTS): 20

Keywords:

circulating tumour cells
CTCs
metastatic breast cancer
size-based filtration
platelets

Pages: 51

+ appendix: 15

Stavanger, 29.05.2022
date/year

Bachelor's Thesis

Platelet Interaction with Circulating Tumour Cells Isolated by Size-based Filtration from Patients with Metastatic Breast Cancer

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Abstract

Metastasis is the main reason for cancer associated deaths. Circulating tumour cells (CTCs) play a large role in the metastatic process and in the progression of cancer disease. There is evidence suggesting that platelet interaction with CTCs may facilitate in metastasis. The purpose of this project was to use size-based filtration to enrich CTCs from patients with metastatic breast cancer and analyse them for the presence of platelets on their surface. The hope is that the project will contribute to a better understanding of platelet-CTC interaction.

Cell line culturing of ZR75-1 cells was used for validation of immunofluorescent staining with various fluorophores and enrichment experiments. Validation of CTC enrichment was done with VYCAP's size-based filtration technology using EDTA respectively CellSave blood. Microscopy was used to validate the results of the staining and to enumerate the cells. The observations show that filtration caused damage to the samples, particularly fresh cells. Fixed cells tolerated the filtration better and resulted in slightly higher recoveries in validation experiments.

CTCs were isolated from patients with metastatic breast cancer using size-based filtration. 10 blood samples from 7 individuals were filtrated to isolate CTCs and analyse them for the detection of platelets. There were detectable CTCs in 9 out of the 10 patient samples, with some of the enriched CTCs interacting with platelets. Out of 9 patient samples with detectable CTCs, 6 samples also contained CTCs with detectable platelets. The number of CTCs with platelets varied between individuals, with the percentage of CTCs with platelet interaction ranging from 5,7% to 70%. The percentage of CTCs that interacted with platelets out of the total number of CTCs was 25,5%. Apart from single CTCs, the enrichment also revealed CTC-clusters in some samples. All the CTC-clusters that were found had detectable platelets in them. These observations suggests that CTC-clusters are more often associated with platelets than single CTCs.

The conclusion drawn from the project is that it is possible to detect platelets in CTCs enriched from patients with metastatic breast cancer using size-based filtration. However, more patient samples are needed in order to give a reliable estimate of the frequency of interactions between CTCs and platelets.

Acknowledgements

I would like to express my greatest gratitude to professor Oddmund Nordgård, who has been my supervisor during this project. He has been very engaged in the project, listened to my ideas, and helped me to implement them in practise. His enthusiasm for this field of research and his ability to convey the subject has motivated me through the project. He has been more than patient with me and my questions, great at explaining things and he is extremely dedicated to help. He is one of the best teachers I have ever had and certainly the only one who has helped me by using a scalpel to operate on an adapter to adjust it so it can fit into a microscope.

I would also like to thank my fellow classmate Julie Kloster Snekkevik for our collaboration during this project. I would like to thank Morten Lapin, for showing me the VYCAP equipment and for answering questions and helping me to finding solutions to problems. An additional note of appreciation to Satu Sinikka Oltedal for offering help in the laboratory, answering to questions, and sharing the centrifuges. I would like to thank Kjersti Tjensvoll for taking interest in my studies and discussing possibilities for the future.

Thanks to Irene Tveiterås Øvestad, Kristin Aanestad, and Siv Janne Christensen Salomonsen who has helped with collection of blood samples. My sincerest gratitude goes to everyone who has donated blood.

Finally, I am grateful to all the personnel at the Laboratory for Molecular Biology, Department of Haematology and Oncology at Stavanger University Hospital, for creating such a lovely work environment and for welcoming us into the laboratory. A special thanks goes to the people at pathology who has shared the microscope with us.

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Abbreviations

BSC	Biological Safety Cabinet
CAFs	Cancer-Associated Fibroblasts
CTCs	Circulating Tumour Cells
CTC-C	Circulating Tumour Cell Cluster
CTC-S	Single Circulating Tumour Cell
DCIS	Ductal Carcinoma In Situ
ECM	Extracellular Matrix
EMT	Epithelial- to Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ER+	Oestrogen Receptor Positive
ER-	Oestrogen Receptor Negative
HR+	Hormone Receptor Positive
HR-	Hormone Receptor Negative
IF	Immunofluorescent
KRT	Keratin
KRT+	Keratin Positive
LCIS	Lobular Carcinoma In Situ
mBC	Metastatic Breast Cancer
MDSCs	Myeloid-Derived Suppressor Cells
MINDEC	Multimarker Immunomagnetic Negative Depletion Enrichment of CTCs
PBMCs	Peripheral Blood Mononuclear Cells
PPE	Personal Protective Equipment
PR+	Progesterone Receptor Positive
PR-	Progesterone Receptor Negative
RBC	Red Blood Cell
SUS	Stavanger University Hospital
TAMs	Tumour-Associated Macrophages
TANs	Tumour-Associated Neutrophils
WBC	White Blood Cell
WHO	World Health Organisation

1. INTRODUCTION

1.1 BREAST CANCER

With an impact on many lives, breast cancer continues to be a large field of research. According to the World Health organisation (WHO), there were 2 261 419 new incidences of breast cancer worldwide in 2020 [1]. This corresponded to 11.7% of all new cases of cancer that year. During the same year there were 684 996 (6.9%) deaths from breast cancer [1]. Most of the deaths were due to metastatic spread of the disease. Breast cancer is one of the most common types of cancers in Norway, with 3455 new cases in 2020 [2].

1.1.1 Risk Factors

The number one risk factor for developing breast cancer is age [3] and having a female gender. Other risk factors for breast cancer may be divided into two categories: inherited factors and acquired factors.

Breast cancer primarily occur in females and inherited factors plays a large role in the development of breast cancer, compared to other types of cancer in which genetics are only connected to 5-10% of cases [4]. There are around 30 genes known to be related to breast cancer [5]. It has been observed that 50% of cases of familial breast cancer are associated with germ line mutations in the BRACA1 and BRACA2 genes [3]. Other genes frequently mutated in breast cancer are for example PTEN, ATM and TP53 [5]. Gender-related aspects of the disease include pregnancy history, onset of menstruation as well as onset of menopause [3].

Acquired risk factors are connected to lifestyle and environment and may consist of for example nutritional aspects and exposure to certain chemical agents that may induce cancer or increase the risk of developing cancer [3, 6]. It has been observed that the frequency of breast cancer deaths is greater in Northern Europe than in the rest of the world [7]. The fact that breast cancer occurs more frequently in the northern latitudes could be a combination of environmental, lifestyle and genetic factors. An unhealthy lifestyle poses a higher risk of developing breast cancer. Lifestyle-related risks are for example smoking, obesity, physical inactivity, and high alcohol consumption [8].

1.1.2 Diagnosis and Classification of Breast Cancer

Breast cancer is a type of cancer that starts in the tissue of the breast, most often in the cells lining the milk ducts [9]. Initial detection of breast cancer is done by sensation of a lump under the skin, the diagnosis is confirmed by imaging and biopsy. Commonly, it is the patient or their physician that detect the lump [10]. Further examinations are made in order to present a diagnosis.

Triple diagnostics are used when breast cancer is suspected. This consist of clinical examination, image diagnostics and biopsy. All tumours should be histologically confirmed by needle biopsy or vacuum biopsy before surgery [2]. Usually, a mammography is taken in combination with an ultrasound and a tissue biopsy [10]. Regular screening programs with mammography can detect breast cancer before it can be noticed as a lump.

1.1.2.1 Subtypes of Breast Cancer

One part of the process of diagnosis is to find out what type of breast cancer that the tumour is composed of. Breast cancer may arise in different parts of the breast and thus there are several types of breast cancer. Ductal breast cancer being the most common type, other types include HER2-positive breast cancer and triple negative breast cancer [10]. Subtypes of breast cancer are categorised according to expression of the oestrogen hormone receptor (ER) and progesterone hormone receptor (PR). It is also dependent on HER2 – a receptor tyrosine kinase, and the molecular marker Ki-67 which plays a role in proliferation. Breast cancers can be hormone receptor positive (HR+), meaning that they overexpress both the oestrogen receptor and the progesterone receptor, or hormone receptor negative (HR-), meaning that they express low levels of the oestrogen and the progesterone receptors [11]. Breast cancers are also classified by molecular subtype, based on gene expression profiling. There are 5 main molecular subtypes of breast cancer, (see Figure 1). The most common type is called Luminal A, comprising 40% of all cases. Luminal B constitutes 20% of cases, and is more aggressive than Luminal A. While Luminal A has low levels of Ki-67, Luminal B has high levels of this marker. Both Luminal A and B are hormone receptor positive. Normal-like breast cancer is also HR+ and has a lower survival rate than Luminal A. HER2-enriched breast cancer is HR- but is positive for the HER2 receptor (HER2+). This subtype has a faster growth rate than the luminal subtypes. The most aggressive subtype of breast cancer is triple negative breast cancer, which is associated with mutations in the BRCA1 gene [12].

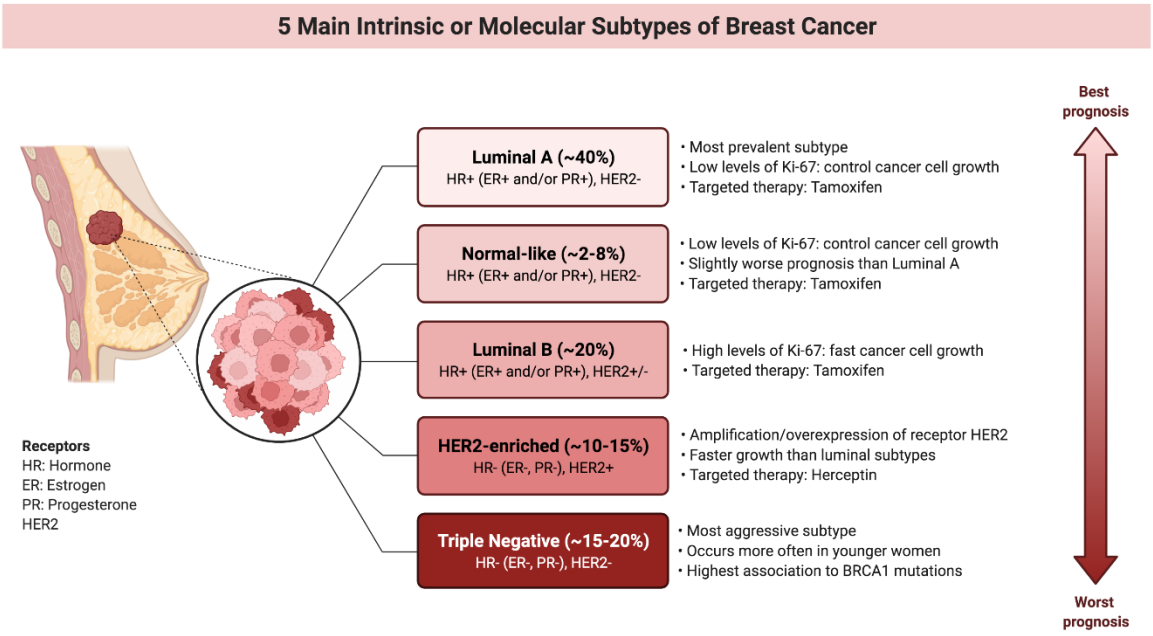


Figure 1. Five Main Intrinsic or Molecular Subtypes of Breast Cancer. The five subtypes of breast cancer are presented in order from best to worst prognosis. The subtypes are derived from their constitution of hormone receptors and their levels of Ki-67. HR: Hormone receptor, ER: Oestrogen receptor, PR: Progesterone receptor. + indicates positive, - indicates negative. Reprinted from "Intrinsic and Molecular Subtypes of Breast Cancer" by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

1.1.2.2 Stages of Breast Cancer and TNM-Classification

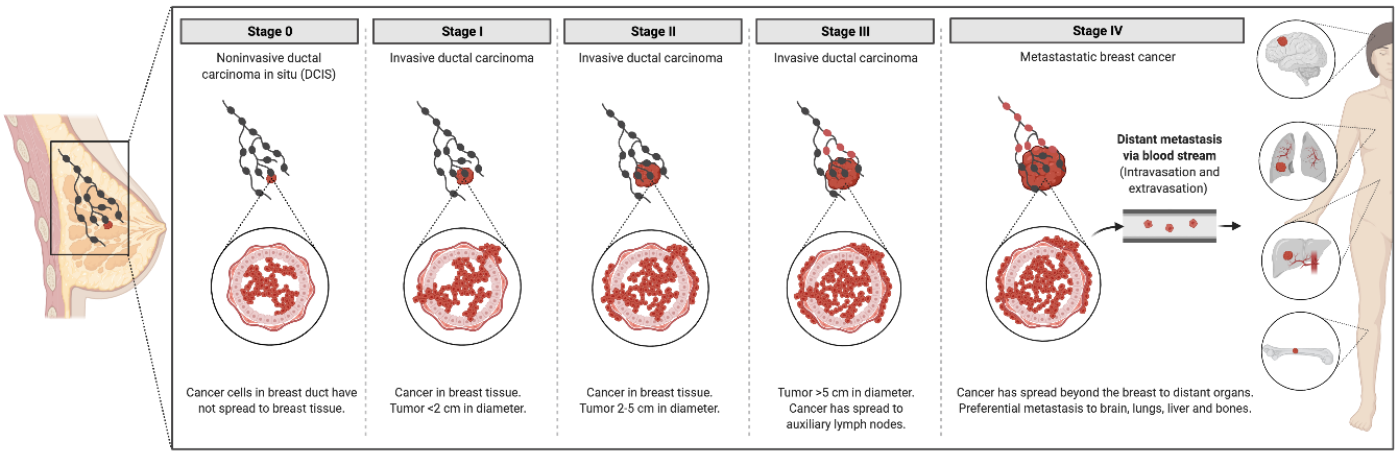


Figure 2. Stages of Breast Cancer. The stages of breast cancer reach from stage 0 to stage IV and they reflect the progression of the disease. Stage 0 describes a non-invasive carcinoma in situ, stages I-III describes varied grades of developed disease and stage IV a fully blown disease with spread to other parts of the body. The figure is adapted from "Stages of Breast Cancer", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

Breast tumours can be divided into benign and malignant. Benign tumours are pre-cancerous lesions such as ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) depending on which part of the breast is afflicted. These tumours show cellular abnormalities but are still confined to the normal localisation of the breast epithelium. A malignant tumour is invasive and infiltrates surrounding tissue.

Breast cancer stages are based on the size of the tumour, localisation of the tumour and invasiveness of the tumour, reaching from stage 0 to IV (see Figure 2). Malignancies are also further classified according to the TNM system, that is based on involvement with lymph nodes and metastasis [13, 2]. Breast cancer staging is done according to international guidelines and the TNM classification system is used worldwide. TNM stands for:

- T = **T**umour size
- N = Lymph **N**ode
- M = **M**etastases

For an overview of the stages of breast cancer and their corresponding TNM-classification, see Table 1 below. For an overview of T-staging, see Appendix A. Each of the stages can be divided further depending on how they affect the patient (see Table 1). The pre-stage, or stage 0, describes a non-invasive tumour – so called cancer in situ, where the tumour only grows where it first arose. In the first stage of breast cancer the tumour starts to grow into the tissues of the breast and become invasive. Stages I-III describes an invasive carcinoma with different sizes of the original tumour and with potential spread to auxiliary lymph nodes. When the tumour is larger than 5 cm or has grown into the skin or chest the cancer has reached the third stage [10]. Stage four describes an invasive tumour that has metastasised to other parts of the body. One of the characteristics of metastatic breast cancer is organ-specific spread, meaning that the cancer metastasises to specific organs. Metastatic breast cancer often metastasises to the brain, bones, liver, and lungs [14]. The main cause of death in patients diagnosed with breast cancer is metastasis [15].

Table 1. Stages of Breast Cancer and their Corresponding Clinical Classification in the TNM-system. Breast cancer is divided into stages reaching from 0 – IV depending on the degree of development of the disease. Breast cancer is also classified according to the TNM-system. This table shows the stages in relation to the TNM-classification, as well as the character of the tumour and if it is resectable or not.

Stage	TNM-Classification	Character (trait)	Resectable
0	Tis N0 M0	Localised within the breast	Yes
IA	T1 N0 M0	Locally invasive	Yes
IB	T1 N1mi M0	Locally invasive	Yes
IIA	T0 N1 M0 T1 N1 M0 T2 N0 M0	Locally invasive	Yes Yes Yes
IIB	T2 N1 M0 T3 N0 M0	Locally invasive	Yes No
IIIA	T0 N2 M0 T1 N2 M0 T2 N2 M0 T3 N1 M0 T3 N2 M0	Locally advanced	No No No No No
IIIB	T4 N0 M0 T4 N1 M0 T4 N2 M0	Locally advanced	No No No
IV	T(any) N(any) M1	Metastatic	No

TNM – Classification:
Tis = Carcinoma in situ
T0 = No primary tumour recognised
T1 = Tumour ≤ 2 cm in diameter
T2 = Tumour > 2.0 ≤ 5.0 cm
T3 = Tumour > 5 cm in diameter
T4 = Tumour is infiltrating the skin or breast wall
N0 = No detectable regional lymph node metastases
N1mi = Lymph node micro-metastasis > 0,2 ≤ 2 mm in diameter
N1 = Auxiliary lymph node metastasis
N2 = Ipsilateral auxiliary lymph node metastasis
M0 = No detected metastasis to other tissues
M1 = Detected metastasis to other parts of the body

1.1.3 Treatment

The treatment of breast cancer varies depending on the disease stage (Section 1.1.2) [10]. After diagnosis it is necessary to determine if the tumour is operable or inoperable, since this will impact the treatment [2]. Primary resectable tumours are seen in stages I and II. Unresectable tumours are seen in stages II-IV (see Table 1 in Section 1.1.2.2). Resectable tumours constitute the majority of breast cancers. Depending on the type of breast cancer, the surgical removal is done in combination with other treatment options. Unresectable tumours can be treated with other methods than surgery.

Drug treatment is a common approach for treatment of breast cancer, also after surgical removal of a tumour. The drugs may consist of cytotoxic chemotherapy, endocrine therapy, or some type of targeted therapy against the specific type of cancer [6, 10]. Hormone receptor status is important to decide treatment [11]. Depending on the subtype of breast cancer it can be treated with targeted therapies such as Tamoxifen which is a drug aimed at the ER, and Herceptin which is aimed at the HER2 receptor protein. Other methods of treatment are radiation, which is frequently used because it lowers the risk of a relapse [10] and bone strengthening treatment. Palliative care is given to patients with incurable disease.

The challenge with treatment is that many of the current treatments are general and do not target the individual tumour of the patient. Side effects from treatment also presents a challenge. A severe problem is that some cancers become resistance to certain therapies [6]. Lately, new therapies have emerged in the form of immunotherapies. These therapies target the immune system of the patients and promotes the immune system to fight cancer cells. Now there are many different kinds of immunotherapies. Notably, checkpoint inhibitors are showing great promise for different types of cancers – including breast cancer [16].

1.2 Circulating Tumour Cells

In 1869 the first description of circulating tumour cells (CTCs) were published by Ashworth, who noticed that some cells in the blood were similar to tumour cells of the patient [17]. Today, we know that circulating tumour cells are produced by intravasation, which is the process where invading tumour cells are entering the blood stream (see Figure 3) [7]. The mechanisms of metastasis are complex and the details of the metastatic cascade are still subject to research [6, 7].

1.2.1 The mechanisms behind circulating tumour cells – metastasis

The process of metastasis is of importance for understanding the nature of circulating tumour cells and the metastatic process consists of several steps that are shown in Figure 3. The steps involve the infiltration of distant tissue, evading immune defences, adaptation to supportive niches and surviving as latent tumour-initiating seeds [15].

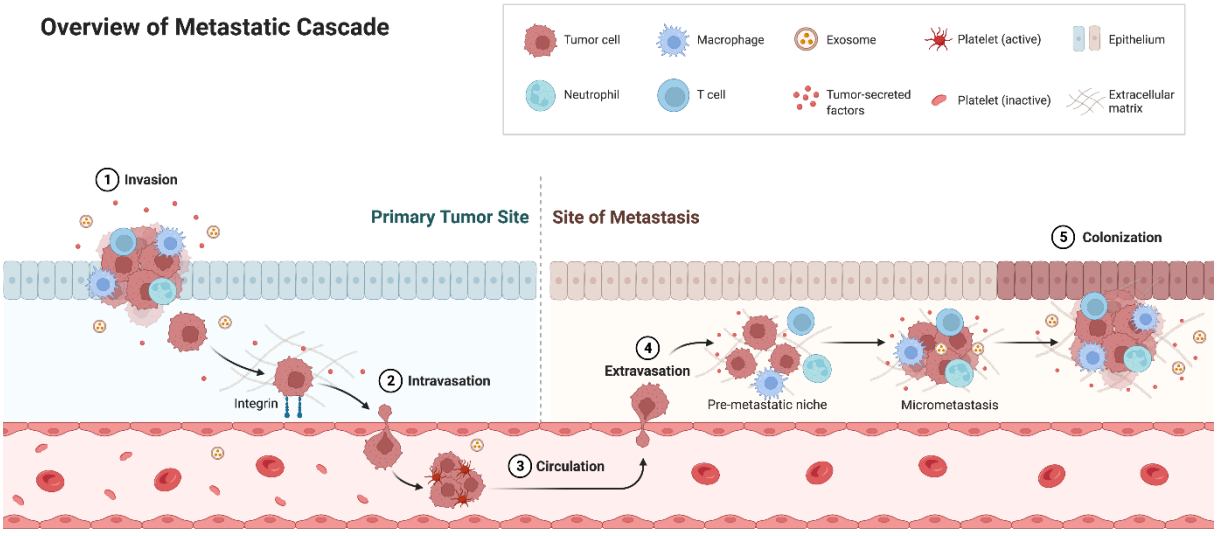


Figure 3. The Metastatic Cascade. The steps of the metastatic cascade include invasion, intravasation, circulation, extravasation, micrometastasis, and colonization. The metastatic cascade is of importance for understanding the background of circulating tumour cells, which are produced during the process of intravasation. Reprinted from "Overview of Metastatic Cascade" by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

The first step in the process of metastasis is local invasion, when a tumour turns malignant. This involves angiogenesis stimulated by angiogenic factors and breakage through the basement membrane of surrounding tissue. In the process of a tumour turning malignant, abnormal cells secrete proteases which degrade the extracellular matrix. The next step is intravasation, which is the entering of the bloodstream. The process of intravasation produces circulating tumour cells. In the

bloodstream, the CTCs can interact with other components of the blood (see Section 1.2.3 and 1.2.4). CTCs are too large to pass the smallest capillaries. This causes arrest in micro-vessels. There are two models for how extravasation occurs. The first model states that a CTC can actively migrate through the endothelial wall of the blood vessel. This is facilitated through biochemical communication with endothelial cells. The other model explains that micro-metastases form inside the capillary. The formation of micro-metastases in other parts of the body is called colonization. The process is very inefficient and micro-metastases seldom expand into macro-metastases [7]. Even if the process is inefficient, a high number of CTCs in the blood has been shown to correlate with a poor prognosis in patients with early and metastatic breast cancer [18, 19, 20]. Deepened understanding of the metastatic cascade of CTCs could aid in development of targeted drugs against cancer metastasis [21].

1.2.2 Biology of CTCs

Distinctive traits of CTC biology make it possible to detect and distinguish them from other cells. It is generally possible to recognize a CTC as a cancer cell since it keeps the distinct characteristics of the tumour cells even after leaving the original tumour site. Although CTCs may undergo various changes in order to intravasate into the bloodstream and colonize in other tissues, CTCs with a different phenotype than the original tumour are rarely observed [22].

Morphological features of CTCs are one of the factors that makes them distinguishable from other cells in the blood. Many of the CTC markers are also present on the cells of the primary tumour. It has been shown that the morphology of many CTCs is analogous to that of tumour cells in cytologic preparations. Several morphologic criteria have been proposed for identification of CTCs, including properties of the nucleus, a high nuclear to cytoplasmic ratio and the presence of 3-dimensional sheets [23]. It is also observed that most CTCs are larger than the cells in the circulatory system. Since CTCs are different from other cells in the blood it enables their enrichment and detection through different methods.

CTCs have their distinct molecular markers that separates them from other cells in the blood. Molecular markers used for detection of CTCs are for example EpCAM, which is the most used, and keratins. The CTC markers often resemble the molecular markers of the primary tumour making it easier to decide which marker to use for detection of CTCs in the blood. There are different CTC-associated markers for different types of cancer and the markers can be targeted towards epithelial or mesenchymal phenotypes as well as specific molecular markers [21]. Molecular markers used to detect CTCs with epithelial origin in breast cancer are for example EpCAM, various keratins such as KRT5, KRT7 and E-cadherin. Mesenchymal markers used for identification of CTCs in breast cancer are vimentin and fibronectin among others and specific markers in breast cancer are for example HER2 and ER [21]. Keratins and E-cadherin are markers of epithelial cells that are often used for detection of CTCs. Much remain to be revealed about the expression of keratin in CTCs and it is not known yet if all CTCs can be detected using antibodies directed against keratin [24].

Most tumours are of epithelial origin and thus the CTCs will reflect this. In early tumours, the cells retain their expression of these epithelial phenotypes. However, in advanced carcinomas the cells exhibit traits that are more mesenchymal [25]. The process of invasion requires a mesenchymal phenotype [26]. As a rule, most CTCs resemble the cells of the primary tumour. However, there are exceptions. Some circulating tumour cells are supposedly able to make a transition from an epithelial phenotype (the phenotype that is most common in the primary tumour) to a mesenchymal phenotype. This is called the epithelial to mesenchymal transition (EMT) and aids the cells in becoming more aggressive and able to extravasate and form distant tumours [7, 25].

Differences between epithelial cells and mesenchymal cells are for example that epithelial cells are less motile, while mesenchymal cells are motile and invasive. The cytoskeleton of epithelial cells expresses keratins in contrast to the cytoskeleton of mesenchymal cells which express vimentin. Epithelial cells form adherent and tight junctions with other epithelial cells. Mesenchymal cells do not form these junctions, instead they attach to the extracellular matrix with focal adhesions [25].

1.2.3 Interaction with leukocytes

CTCs are known to interact with other cells in the circulatory system [26]. CTC associated cells are fibroblasts, leukocytes, endothelial cells, pericytes and platelets [27]. Leukocytes are the cells of the immune system, also called white blood cells (WBC). They are a part of the body's immune defence, and the average lifetime of a WBC is between a few hours up to a few days [28]. There are several different types of leukocytes. For an overview of the different cells in the blood, see Appendix B. The immune system and leukocytes play a role in the fight against cancer. However, cancer cells are known for being able to evade immune detection [7].

There are different types of leukocytes, but the most abundant in humans are neutrophils. The mechanism of neutrophil-interaction with cancer cells in connection to cancer progression has been studied [29]. It has been found that Tumour-associated neutrophils (TANs) and a high neutrophil to lymphocyte ratio in the blood indicates a poor prognosis [21, 29, 30]. TANs also contribute to disease progression as well as angiogenesis and invasion [30]. Findings also suggests that CTCs in interaction with neutrophils in breast cancer patients metastasised more and exhibited altered gene expression of cell cycle progression [31]. Interaction between neutrophiles and CTCs can occur directly through for example through cell-to-cell junctions, but it can also happen indirectly when neutrophiles release proteins and when DNA-histone complexes interact with CTC biology [32].

Tumour-associated macrophages (TAMs) aids in metastasis and extravasation of CTCs through multiple mechanisms. They promote metastasis by changing the constitution of the local matrix, implementing angiogenesis, and inhibiting the immune response that is supposed to kill of cancer cells [33].

CTC clusters with myeloid-derived suppressor cells (MDSCs) seem to evade immune response [21]. The mechanisms are yet to be understood. Cancer-associated fibroblasts (CAFs) constitute a large part of the tumour microenvironment. They take part in angiogenesis, metastasis and even drug resistance. They alter the structure of the ECM and thus help tumour cells to invade surrounding tissue. They can also impact the biology of the CTCs [34].

1.2.4 Interaction with platelets

Platelets, also called thrombocytes, are an essential part of the blood which foremost take part in the coagulation process [35]. There are approximately 250 000 platelets per ml of blood and the lifespan of a platelet is around 10 days [36]. Platelets are produced from larger cells in the bone marrow called megakaryocytes [36, 37]. The morphology of platelets are round or oval-shaped, they lack a nucleus and they are quite small with a diameter of about 2-4 µm [38, 35].

Recent studies suggest a connection between circulating tumour cells and platelets [26, 38]. It is suggested that recruitment and activation of platelets plays a role in cancer progression and metastasis. It seems like platelets can bind to CTCs and facilitate their travel through the circulatory system, postponing their degradation in the blood, and thus giving the CTCs more time to potentially form micro-metastases. Platelets are also hypothesised to protect the CTCs from being targeted by

immune cells for destruction. Platelets are thought to protect CTCs against mechanical stress and help the CTCs become resistant to anoikis [21].

By prothrombotic and proagulent factor releasement, the platelets can bind and form aggregates and attachments to CTCs [21]. Other molecules, such as TGFβ released by platelets, have also been found to have an impact on EMT of CTCs, which in turn leads to invasion and metastasis [21, 39, 40]. Some evidence points towards the expression of thrombin on the surface of primary tumour cells for promotion of metastasis through platelet mechanisms [41].

1.2.5 Clinical Relevance

CTCs potentially have a huge clinical relevance. In 2004 the US Food and Drug administration (FDA), approved one method of CTC detection (the CellSearch system) as a method to monitor cancer treatment. The CellSearch system has since been implemented on many types of cancer, including metastatic breast cancer [42]. The most common use of CellSearch is prognostic classification [43]. CTCs are currently used as biomarkers for many solid cancers [21]. CTCs can be utilized for early diagnosis, evaluation of the prognosis and for monitoring disease progression and respondence to therapy.

The fact that CTCs can be enriched and detected from a blood sample makes this method compelling to use as a tool in the monitoring of patients recovering from cancer. A blood draw is minimally invasive, with little risk of complications. A tumour is only detectable with imaging when it reaches a certain size. However, research points to the fact that CTCs are produced early in tumour development [44]. This means that it would be possible to detect CTCs in the blood before one could see the primary tumour on a scan [45]. The detection of CTCs in blood samples is used as a prognostic factor and the enumeration of CTCs is an accepted prognostic indicator for metastatic and non-metastatic breast cancer, prostate cancer, and colorectal cancer that influences the progression-free survival as well as the overall survival [19, 20, 23]. CTCs may be used as early markers of breast cancer metastasis or to monitor the effect of therapies. Evidence suggest that CTC clusters constitute a greater risk of metastasis compared to single CTCs [23]. Since many of the markers present on CTCs are similar to the ones on the cells in the primary tumour – CTCs can be used to make decisions about treatment [22].

1.2.6 Enrichment and Detection

Due to the low frequency of CTCs in blood, they must be enriched in order to be identified [46]. Enrichment of CTCs can be done based on either physical or biological properties or a combination of the two [47]. The starting point for enumeration of CTCs is a blood sample [45, 48]. CTC enrichment is still under development, and it currently remains a research tool foremost [23]. Enrichment is done based on what is known from the biology of CTCs - the two categories for enrichment being physical properties and biological properties. Furthermore, detection can be divided into cytometric techniques (based on whole cells) and techniques grounded in nucleic-acids [49].

Figure 4 provides a brief overview of some of the most common methods for CTC enrichment. There are however many other methods in addition to these.

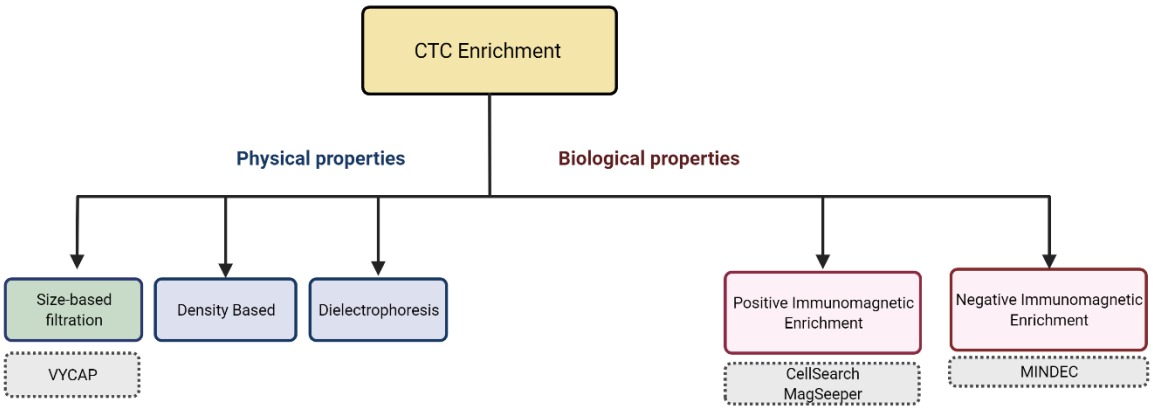


Figure 4. Brief Overview of CTC Enrichment Technologies. CTC enrichment methods can be roughly divided into physical and biological properties. Dotted grey boxed show examples of technologies. This project utilised size-based filtration (marked with green) for enrichment of CTCs in patients with metastatic breast cancer. The figure is created with BioRender.com.

Enrichment that is dependent on biological properties of CTCs can be either positive or negative. Positive enrichment targets markers on the CTCs surface to capture the CTCs directly whereas negative enrichment targets the hematopoietic cells in the blood for depletion of these – indirectly capturing the CTCs [50].

The most used method today is the CellSearch system, which is EpCAM based [51]. It is a standard technique for CTC enrichment that utilizes positive enrichment. An example of a biological property is the expression of protein markers on the surface of the CTCs [52]. Biological selection utilises immunoaffinity of CTCs or leukocytes. Positive selection can be made *ex vivo* or *in vivo*. Selection *in vivo* involves the capturing of CTCs directly in the bloodstream. Examples of selection *ex vivo* are the CellSearch system, MagSweeper and CTC chip. Examples of technologies for *in vivo* selection are CellCollector and Photoacoustic nanodetector [52]. The CellCollector is used in breast cancer patients and it works by an intravenously placed EpCAM coated wire that collects CTCs [42].

There are also different methods for negative depletion. Negative depletion by the MINDEC method relies on selection and depletion of leukocytes by several different antibodies:

- Anti-Hu CD45, 13-0459-82, x2
- Anti-Hu CD16 Biotin, 13-0168-82, x4
- Anti-Hu CD235a Biotin, 13-9987-82, x4
- Anti-Hu CD19 Biotin, 13-0199-82, x2
- Anti-Hu CD163 Biotin, x1

The combination of multiple markers covers and targets a large part of the different cell types present in blood, resulting in maximal depletion of PMBCs [17].

Enrichment based on physical properties are reliant on aspects such as the size of CTCs or the charge of the cells. The majority of CTCs are larger than blood cells. It is also thought that CTCs are less deformable [52], although some research suggest that CTCs which have undergone a change in phenotype may be more deformable than blood cells [53]. Size-based filtration was used in this project, marked with green in Figure 4.

After enrichment of CTCs, detection of the cells can be performed using various techniques. Cytometric techniques can for example be visual confirmation of CTCs using antigen expression and fluorescently label the cells. Cytometric techniques allow for further analysis and classification of the morphology of the cells. Detection techniques grounded in nucleic-acids, such as RT-PCR or qRT-PCR, identify CTCs based on their genetic or epigenetic alterations. In breast cancer, the detection of mRNA of overexpressed factors is often used [49].

1.3 PURPOSE

The primary aims of this project have been to:

- Establish a method for CTC enrichment using size-based filtration
- Investigate to which extent there are platelets bound to CTCs in patients with metastatic breast cancer.

More specifically, the primary aims of the project have been to test and validate the VYCAP filtration system for enrichment of CTCs on normal blood samples with spiked-in cancer cells and ultimately use the technology on patient samples to detect platelets on CTCs.

Secondary aims have been to establish and optimise a protocol for immunofluorescent staining of platelets with CD61 antibody and to validate the staining on normal blood samples with and without spiked-in cancer cells. In addition, optimalisation of protocols for immunofluorescence-based detection of CTCs was performed. The goal of optimising the immunofluorescence detection was to be able to distinguish between CTCs, leukocytes, and platelets in the same sample. The optimalisation of immunofluorescence protocols was done in collaboration with Julie Kloster Snekkevik. While this project has been using size-based filtration for enumeration of CTCs, her project has been using the MINDEC method.

The long-term purpose of this project is to increase the knowledge about platelet interaction with CTCs in patients with metastatic breast cancer to determine its clinical impact and potential utility. Hopes are that this project can contribute to a better understanding of the metastatic process, the interaction between platelets and CTCs, and possible treatment options based on this.

2 MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 Cell Line

The cell line used for this project was ZR75-1 (ATCC CLR1500), derived from the mammary gland tissue of a ductal breast carcinoma in a 63-year-old female [54].

2.1.2 Reagents and Equipment

The following reagents have been applied in the experiments, see Table 2.

Table 2. Overview of reagents used in experiment.

Material	Manufacturer	Catalogue Number	Application
RPMI-1640	Sigma-Aldrich	R0883-500ML	Cell culture
0.25 % Trypsin-EDTA	Sigma-Aldrich	T4049-500ML	Cell culture
Fetal Bovine Serum	Sigma-Aldrich	F7524-500ML	Cell culture
Glutamine 200 mM	Sigma-Aldrich	G7513-100ML	Cell culture
Penicillin-Streptomyocin Antibiotics 10 mg/ml	Sigma-Aldrich	P4433-100ML	Cell culture
Bovine Serum Albumin	Sigma-Aldrich	A7030-50G	CTC enrichment
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	D8537-500ML	Cell culture (splitting)
Trypan Blue Stain 0.4%	Invitrogen	T10282	Cell-counting
Paraformaldehyd in PBS	Sigma-Aldrich	158127	Fixation
Anti-Human CD45 antibody, APC	Invitrogen	17-0459-42	Staining, APC
Anti-Pan cytokeratin antibody, Clone: AE1/AE3, eFluor 570	Invitrogen	41-9003-82	Staining, keratin
Anti-Human CD61, REAfinity™, Clone REA761	Miltenyi Biotec	130-110-748	Staining, FITC (platelets)
Pan Cytokeratin Monoclonal Antibody (C-11), PE	Invitrogen	MA5-28574	Staining, keratin
DAPI	Sigma-Aldrich	32670-25mG-F	Staining, nuclei
FcR Blocking Reagent human	Miltenyi Biotec	130-059-901	Staining
Lymphoprep™	StemCell Technologies	07851/07861. 1114547?	Density gradient centrifugation
0.5 M EDTA	Merck	1 084 180 250	Isolation of platelets
Prolong Diamond Antifade Mountant	Thermo Fisher Scientific	P36965	CTC enrichment
Saponin 10 %	Sigma-Aldrich	47036-50G-F	Staining
Phosphate Buffered Saline (PBS)	Sigma-Aldrich	P4417-100TAB	CTC enrichment

The equipment used for experiments is listed in Table 3.

Table 3. Overview of equipment used for experiments. NA: not available.

Equipment	Manufacturer	Catalogue number	Application
Hera Cell 150	Heraeus	NA	Incubator
Hera Safe	Heraeus	50073961	Biological Safety Cabinet, cell culture
Megafuge 1.0R	Heraeus	NA	Centrifugation
Allegra X-30R Centrifuge	Beckman Coulter™	NA	Centrifugation
Countess II	Thermo Fisher	NA	Cell-counting
Countess cell counting chamber slide	Thermo Fisher	C10283	Cell-counting
Micro tubes, 1.5 ml	Sarstedt	72690001	Staining, cell-counting, etc.
Tube 50 ml (with cape)	Sarstedt	62559001	Solutions
Tube 50 ml (without cape)	Sarstedt	62547254	Centrifugation
Tube 15 ml	Sarstedt	62554502	Centrifugation
EDTA blood collection tubes 9 ml	Vacuette® Tube	455036	Collection of blood samples
CellSave Preservative tubes 10 ml	CellSearch®	7900005	Collection of blood samples
SepMate™-50	Stemcell technologies	85450	Density gradient centrifugation
Pump Unit	VYCAP	PU-500	CTC enrichment
MicroSieves	VYCAP	FS-510	CTC enrichment
Staining holder	VYCAP	SH-60	CTC enrichment, permeabilization and staining
Olympus CKX31	NA	NA	Microscopy

2.1.3 Prepared Solutions

PBS, 0.01 M:

- 5 PBS tablets
- 1000 ml of mQ water

EDTA, 0.5 M:

- 186.1 g of disodium EDTA•$2H_2O$
- 800 ml of mQ water
- pH adjustment to 8.0 by addition of NaOH (5 M or 10 M)

Saponin, 10%:

- 5 g saponin
- 50 ml of mQ water
- Slightly heat to dissolve
- Filter through a 0.2 µm filter

Paraformaldehyde, 4%:

- 2 g paraformaldehyde
- 50 ml PBS
- Warm to 65° to dissolve
- Filtrate through 0.22 µm filter

DAPI:

- 5 mg DAPI
- 1 ml mQ water

IF Staining Buffer (50 ml 0.5% BSA, 2 mM EDTA):

- 0.25 g BSA
- PBS to 50 ml
- 200 µl 0.5 M EDTA

IF Staining Buffer with 0.1% saponin:

- 10 ml of IF Staining Buffer
- 100 µl of Saponin 10%

VYCAP buffer PBS/1% BSA, 50 ml:

- 5 g BSA
- 50 ml PBS
- Filter through a 0.8 µm filter

2.1.4 Blood samples

Blood samples were collected from healthy donors for use in optimalisation of protocols and for validation of CTC enrichment. Blood draws were made in both EDTA blood collection tubes and CellSave blood collection tubes.

In total, 10 blood samples were acquired from 7 patients with metastatic breast cancer. The patients were recruited to an observational study at Stavanger University Hospital (SUS) called “Monitoring of advanced breast cancer using liquid biopsies” (abbreviated MBC-study). The study has been approved by the regional ethical committee (123826) and participation is based on written informed consent. The first three patient blood samples were drawn in EDTA blood collection tubes, and the rest in CellSave blood collection tubes.

When drawing blood, both from volunteers and patients – the first millilitres of blood was discarded. This was done to avoid contaminating epithelial cells and to avoid activation and aggregation of platelets.

2.2 METHODS

2.2.1 Cell Culture

The following techniques were used for work with the ZR75-1 cell line.

2.2.1.1 Aseptic Technique

The purpose of using aseptic technique was to avoid contamination of the cell culture with bacteria, fungi and mycoplasm as well as cross-contamination with other cell lines. The protocols related to cell culturing were followed using aseptic technique. The procedures were done in a cell culture room, where personal protective equipment (PPE) was used in the form of sterile gloves, a dispensable gown, and overshoes. The work with cell line cultures was performed in a biological safety cabinet (BSC). The safety cabinet provided a sterile, particle-free environment by constant airflow. In addition to this, the safety cabinet was sterilized before and after each procedure with 70% ethanol and UV light. For each procedure, the outside of all containers and equipment were sterilized before they were placed inside the BSC. Gloves were sterilised with 70% ethanol prior to placing the hands inside the BSC and beginning the work.

2.2.1.2 Resuscitation of cell line

The cells were stored in a nitrogen tank at -196 °C and resuscitated by quickly thawing them to 37 °C, (see Protocol I below). The ZR75-1 cells were used for spiking of blood samples and to validate enrichment methods. The medium formulation used for the ZR75-1 cell line was:

- RPMI 1640
- 10 % FBS
- 200 mM Glutamine
- Penicillin-Streptomycin 10 mg/ml

The ZR75-1 cells were grown in T75 bottles.

PROTOCOL I: RESUSCITATION OF CELL LINE

This procedure is used to resuscitate a frozen cell line.

1. Preheat the medium needed for the cell line in question, typically added 10% FBS, 2mM Gln, 1x Pen-Strep antibiotics. Preheat to 37°C.
2. Turn on the UV light in sterile bench and cell lab (> 30 minutes)
3. Sterilise the bench, bottles, and gloves with 70% ethanol and be careful to use aseptic technique in the BSC during further work.
4. Transfer 25 ml of preheated medium to a T75 bottle.
5. Take up an ampoule of cells from the nitrogen tank and thaw quickly by keeping the bottom of the pipe in a 37°C water bath. Stop while there is still a small amount of ice left in the tube.
6. Take the ampoule into the BSC, wipe the cap with 70% ethanol and transfer the contents of the T25 bottle gently (drop by drop) with a 2 ml pipette.
7. Incubate the cells into a 37°C incubator with 5% CO_2.
8. Split the cells after 2 days or when they are confluent. Use Protocol II for trypsinisation and splitting of cell line.

2.2.1.3 Subculturing

ZR75-1 is an adherent cell line, where the cells attach to the bottom of the flask. It was observed that the cells did not spread out over the whole bottom of the flask. Instead, they grew on top of each other and formed thorns of cells. The ZR75-1 cells had a slow growth rate and did not need to be split more than twice or three times per week. The following protocol was used for the subculturing of the ZR75-1 cell line.

PROTOCOL II: TRYPSINISATION AND SPLITTING OF CELL LINE

With this procedure ZR75-1 cells are split.

1. Preheat medium, PBS and Trypsin-EDTA to 37 °C. Turn on the UV light in a BSC and let it be on for 30 minutes.
2. Examine the cells under an inverted microscope to verify their shape and confluency to determine if they are ready to be split. In addition, confirm the absence of contaminants.
3. Carefully remove the medium. Add 10 ml of preheated PBS (without Ca2+/Mg2+) and rinse the cells by turning the flask. Avoid adding the PBS directly on the cells as it can cause them to come off. After washing the cells, remove the PBS and discard it together with the used medium.
4. Add 2 ml of Trypsin-EDTA to the flask, close it and rotate the flask to cover the whole bottom with trypsin. Place the flask in the CO_2 incubator for 3-5 minutes.
5. Check that the cells have detached from the bottom.
6. Add an excess of medium and mix thoroughly with a pipette.
7. Add 25 ml of medium to a new T75 flask.
8. Transfer a suiting volume of cell suspension to the new T75 flask. ZR75-1 cells are sub-confluent and a 1:3 dilution is often suitable. The dilution factor is dependent on how fast the cells grow and how long it is to the next subculturing.
9. Place the lid and mix the cells by tilting the flask. Incubate at 37°C and 5% CO_2.

2.2.2 Cell Counting

Cell counting was performed using a Countess™ automated cell counter machine that counts cells automatically following Protocol III below.

PROTOCOL III: CELL COUNTING WITH COUNTESS™ AUTOMATED CELL COUNTER

This procedure is used for counting of PBMC or cell lines in the Countess™ automated cell counter machine.

1. For counting of cell line, stain cancer cells with Typan Blue in a 1:1 ratio (50:50 µl). For counting of PBMC prepare a solution with 45 µl of PBS + 5 µl of cell-suspension + 50 µl 0.4% Trypan Blue.
2. Transfer a small amount (≈10 µl) of the solution to a Countess™ chamber slide and set it to rest for 30 seconds.
3. Insert the slide into the machine.
4. Choose the appropriate setting before starting the count. For counting of cell line choose “cell line”, for counting of PBMC set it to “PBMC”.
5. Start the count. The Countess™ automated cell counter provides the concentration of alive and dead cells.

2.2.3 Isolation and staining protocols

The following protocols were used to isolate and stain white blood cells, cancer cells, and platelets.

PROTOCOL IV: ISOLATION OF PBMC AND PLATELETS

With this procedure both peripheral blood mononuclear cells (PBMCs) and platelets are isolated from the same blood sample with density gradient centrifugation.

1. Transfer the blood sample to a 50 ml tube and add 9 ml of PBS (room tempered). Use about 3 ml of the 9 ml to rinse the blood sample tube. Mix well.
2. Add 15 ml of Lymphoprep™ to a SepMate tube, under the filter.
3. Carefully transfer the PBS-blood-mix to the SepMate tube with Lymphoprep™. Orient the tube in order to avoid adding the blood suspension directly to one of the holes in the plastic filter.
4. Centrifuge the tubes at 800xg for 30 minutes (without brake) and room temperature, if the blood sample is fresh (<2 hours) it is enough to centrifuge for 20 minutes (without brake).
5. Remove most of the plasma fraction with a 10 ml pipette and temporarily store it in a marked 15 ml tube. Discard the plasma if it is not to be used further. If lumps of mononuclear cells are attached to the wall of the tube, they should be released using a pasteur pipette or similar.
6. Carefully, but firmly, pour the rest of the liquid over the plastic filter into a new 50 ml tube. Do it in a quick movement and do not leave the tube too long upside down, as it will cause the red cells to move.
7. Wash the cell pellet with a room-tempered PBS solution filled to 40 ml in total. Resuspend the pellet in the washing solution.
8. Centrifuge 10 min at 200xg and room temperature (moderate brake effect 6/9). Pour the supernatant into a new 50 ml tube. The supernatant contains platelets.
9. Wash the pellet (PBMC) with about 40 ml of PBS. Centrifuge at 200xg for 10 minutes at room temperature. Remove the supernatant, the last drop hanging in the opening of the pipe should be removed with a pipette.
10. Add 1 ml of PBS and resuspend the cell pellet.
11. Take out 5 µl of cell-suspension for cell counting and centrifuge the rest of the suspension for 10 minutes at 300xg.
12. Count the cells (use Protocol III). (Remember that the suspension is a 10x dilution when calculating the concentration).
13. Remove the tubes from the centrifuge. Remove the supernatant with a pipette. Resuspend the cells in a suiting buffer and volume for the next application.
14. Add 0,5 M EDTA to finally 10 mM to the supernatant from step 9 to avoid aggregation of the platelets. Centrifuge for 10 minutes at 1000xg to sediment the platelets (low brake 2). Gently pour the supernatant off.
15. Gently resuspend the pellet in 5 ml of room-tempered PBS with 10 mM EDTA, centrifuge for 5 minutes at 1000xg at room temperature (fast acceleration 9, brake 2).
16. Discard the supernatant and resuspend the pellet in 0.5 ml of PBS added to 10 mM EDTA or any other suitable volume, depending on application.

PROTOCOL V: IMMUNOFLUORESCENT STAINING OF CANCER CELLS AND PBMC

In this protocol, cancer cells and PBMCs are stained with antibodies. The procedure can be used on patient blood samples to stain CTCs. It can also be used for staining of cancer cells in spiking experiments.

1. Isolate PBMCs from blood sample(s) according to Protocol IV.
2. Count the cells using Countess™ (see Section 2.2.2) and sample the desired number of cells into a microtube.
3. If used for spiking experiment, spike the cell into the microtube before proceeding in the protocol.
4. Centrifuge the microtube for 10 min at 300xg. Remove the supernatant with a pipette and resuspend the cells in 50 µl of PBS. Add 50 µl of 4 % paraformaldehyde in PBS and mix the suspension and incubate for 20 minutes in room temperature. In this step the cells are fixed. After fixation, add 1 ml of PBS and centrifuge for 10 min at 300xg.
5. Remove the supernatant with a pipette and resuspend the pellet in 40 µl of staining solution with 0,1% saponin. Thereafter add 10 µl FcR blocking reagent and mixed well. Add the following to the microtube:

- 1:100 concentration of 0,1 mg/ml DAPI
- 1:50 concentration of Anti-Human Pan Cytokeratin AE1/AE3 eFluor 570 (eBioscience, 41- 9003-82, clone AE1/AE3)
- 1:50 concentration of Anti-CD45 APC (eBioscience, 17-0459-41, clone HI30)

Mix well but carefully with a 100 µl pipette. Incubate the microtube for 20 minutes in room temperature without light.

6. Wash the cells by adding 1 ml staining solution with saponin to the tube and then centrifuge 10 minutes at 300xg. Remove the supernatant and resuspend the cells in 10 µl staining solution with a pipette. Add 40 µl of mounting medium and mix well. Transfer to an object glass and place a cover glass on top of it. Apply nail polish on the edges. Keep the slide in a refrigerator (dark).

PROTOCOL VI: STAINING OF CANCER CELLS AND PLATELETS WITH ANTIBODIES AGAINST KERATIN AND CD61

This protocol can be used to stain PBMC and CTCs/spiked in cancer cells on normal object glasses.

1. Leukocytes and platelets should be isolated from obtained blood sample(s) using density gradient centrifugation (Protocol IV, step 1-8, where the centrifugation in step 8 is done at 300xg instead of 200xg for 10 minutes).
2. Resuspend the pellet (after 300xg centrifugation) in 1 ml of PBS and count cell density in a 1x dilution. Transfer a volume of cell suspension that is suitable for the purpose of the experiment to a microtube.
3. If spiking is done, the spiked cells should be added to the microtube from step 2 at this point. Otherwise proceed directly to the next step.
4. Centrifuge the microtube for 10 min at 300xg. Use swingout rotor, which will cause the pellet to end up at the bottom of the tube. The pellet is now so small that it hardly appears. But one can assume that it is at the bottom of the pipe and be careful not to pipette up the last 3-5 µl at the bottom when the supernatant is removed.

5. Remove the supernatant with a pipette, leaving about 3-5 µl of residual fluid, and resuspend the cells gently. Immediately add 50 µl of 4% paraformaldehyde in PBS, mix and incubate for 20 min at room temperature. In this step, the cells are fixed. Afterwards, add 1 ml of PBS and centrifuge the microtube for 10 min at 300xg, with swingout rotor. During centrifugation, make a master mix consisting of IF Staining Buffer, FcR and DAPI. Mix well:

(a) 40 µl IF Staining Buffer (PBS with 0.5% BSA and 2 mM EDTA and 0.1% saponin)

(b) 10 µl FcR blocking solution

(c) 1:100 concentration of DAPI 0.1 mg/ml.

6. Remove the supernatant from the tube but save the last 3-5 µl. Resuspend the cells in 50 µl of the mastermix from the previous point. Then add the antibodies to the tube. Mix well.

    - 1:50 CD45
    - 1:100 AE1/AE3
    - 1:10 CII
    - 1:50 CD61

7. Incubate the microtube for 20 minutes at room temperature without light.
8. Wash the cells by adding 1 ml of IF staining solution without saponin to the tube and then centrifuge for 10 min at 300xg (swing-out). Remove the supernatant and resuspend the cells in the residual fluid. Add 15 µl of mounting medium and mix well. Transfer the content of the microtube to an object glass and place a cover glass on top of it. Apply nail polish along the edge. Store in the fridge (dark).

PROTOCOL VII: ISOLATION, FIXATION AND STAINING OF PLATELETS

With this procedure, platelets are stained with CD61 antibody. The protocol was used for titration of the CD61 antibody to find the optimal concentration for titration.

1. When drawing blood, the first millilitres of blood should be discarded to avoid contaminating epithelial cells and activation of platelet-aggregation.
2. Centrifuge the blood sample tube(s) for 20 minutes at 200xg and room temperature (without brake).
3. Gently transfer 2/3 of the plasma fraction (plate-rich plasma) to a 15 ml tube with a pipette.
4. Centrifuge for 10 minutes at 200xg and room temperature (without brake), to precipitate the remaining cells and cell residues. Transfer the plasma fraction to a new 15 ml tube.
5. Centrifuge for 10 minutes at 900xg and room temperature (without brake). Here, platelets are precipitated.
6. Discard the supernatant and resuspend the pellet in 2 ml of PBS with 10 mM EDTA. Resuspend gently.
7. Transfer 15 µl of platelet suspension into an Eppendorf tube.

8. Resuspend the plates in PBS and then add 50 µl of 1% paraformaldehyde to PBS. Mix and incubate for 20 min at room temperature. In this step, the platelets are fixed. Afterwards, add 1 ml of PBS and centrifuge the tube for 10 min at 1200xg, still with the swingout rotor. During centrifugation, master mix is made consisting of Staining Buffer, FcR and DAPI. Mix well.

(a) 40 µl IF Staining Buffer (PBS with 0.5% BSA and 2 mM EDTA and 0.1% saponin)
(b) 10 µl FcR blocking solution
(c) 1:100 concentration of DAPI 0.1 mg/ml.

9. Remove the supernatant from the microtube with platelets, saving only the last 3-5 µl. Resuspend the platelets in 50 µl of the master mix from the previous point. Then add antibodies to the tube:

    - 1:50 CD45
    - 1:100 AE1/AE3
    - 1:50 CD61

10. Incubate the microtube for 20 minutes at room temperature without light.
11. Wash the platelets by adding 1 ml of IF Staining Buffer without saponin to the tube and then centrifuge for 10 min at 1200xg (swing-out). Remove the supernatant and resuspend the platelets in the residual fluid (about 2-5 µl). Add 10 µl mounting medium and mix well.
12. Transfer to an object glass and place a cover glass on top of it. Apply nail polish on the edges. Store the sample in a refrigerator (dark).

2.2.4 CTC enrichment

In this project size-based filtration was used to enumerate and detect CTCs. This was done with VYCAP.

2.2.4.1 VYCAP

The VYCAP filtering system can be used for CTC isolation and enrichment based on size. The pump unit consists of a tube and a microsieve. The microsieve is composed of a silicon nitride filter with a thickness of 1 µm. The pores of the filter are 5 µm (± 0.2 µm) in diameter and each filter chip contains 160.000 pores [55].

Figure 5. VYCAP Equipment. VYCAP pump unit (left) and microsieve (right).

PROTOCOL VIII: CTC ENRICHMENT WITH VYCAP AND IMMUNOFLUORESCENT STAINING OF CELLS ON A MICROSIEVE

This procedure is used for enrichment and staining of CTCs with the VYCAP equipment. If using fresh EDTA blood, the filtration should be performed within 2 hours. If using CellSave tubes, it is possible to perform the filtration up to 72 hours after the blood draw.

Part 1: Enrichment

1. For filtration of fresh EDTA blood, set the pressure on the filtration pump to 25 mbar or lower. For filtration of CellSave blood, the pressure can be higher – for example 75 mbar. Ideal filtration speed is such that it takes between 1-3 minutes to filter 1 ml of blood.
2. Insert a filtration unit into the pump and check that the o-ring is intact, and that the unit is fully inserted.
3. Transfer as much of the blood sample as possible to the filter unit with a 10 ml pipette.

4. Turn on the pump and wait until the blood is filtered through. Stop the pump just before all the fluid is gone.
5. Wash the blood sample tube with 1 ml PBS/1% BSA and transfer to the filter unit. Make sure to rinse away blood residues from the horizontal edge around the filter.
6. Start the pump and filter through the washing solution. Stop the pump again immediately when all the fluid is gone. Repeat this washing step but add 1 ml of PBS/1%BSA directly to the filter unit this time.
7. Remove the filter unit from the pump and then remove the microsieve part from the filter unit.

Part 2: Staining in microsieve

8. Attach an absorber to a staining holder and place the microsive in the staining holder.
9. Press the upper part of the microsieve down towards the absorbent to remove the remaining fluid on the filter. If liquid remains on the edge, it can be removed carefully with a paper towel. Avoid drying of the filter by proceeding immediately to the next step in the protocol.
10. Wash the filter with 75 µl PBS/1% BSA. Remove the washing solution by pressing down towards the absorber. Repeat the washing.
11. Apply 100 µl of 4% paraformaldehyde in PBS to the filter. Incubate for 20 minutes at room temperature. During this time, make a master mix consisting of 53,5 µl in total for each microsieve, multiply the volumes to make a master mix. Mix well.

    - 40 µl PBS with 0.1% saponin
    - 10 µl FcR blockage solution
    - 0.5 µl 0.1 mg/ml DAPI
    - 0.5 µl Anti-Human Pan Cytokeratin AE1/AE3 eFluor 570
    - 0.5 ul Anti-Human Pan Cytokeratin CK-11 PE
    - 1 µl Anti-CD45 APC
    - 1 µl CD61

12. Remove the paraformaldehyde by pressing down.
13. Wash the filter with 75 µl PBS/1% BSA.
14. Add 53,5 µl of antibody solution with blockage and saponin to the filter. Make sure that the entire filter is covered with liquid. Incubate for 20 minutes at room temperature without light.
15. Remove the staining solution by pressing down onto the absorber.
16. Wash the microsieve with 75 µl PBS/1% BSA and removed the washing solution by pressing down to the absorber.
17. Repeat the washing, but now incubate for 5 minutes at room temperature without light before removing the washing solution. Press extra long against the absorber to remove all fluid.
18. Allow the filter to dry for 10 minutes at room temperature without light in the holder.
19. Turn the filter upside down in the staining holder. Add 15 µl of mounting medium on the back of the filter. Carefully place a VYCAP 8 mm cover glass on top of the filter. Do not press.
20. Turn the filter holder over and carefully apply 13 µl of mounting medium on the top of the filter, approximately in the middle. Gently place an 8 mm cover glass without pressing it down on the filter.
21. Incubate for 10 minutes at room temperature without light. Store in a refrigerator (dark).
22. Look under the microscope and scan the sample in Metafer 4 (Protocol X, found under Appendix C1).

2.2.5 Immunofluorescent staining and Microscopy

Immunofluorescent (IF) staining was used to distinguish between isolated cells. IF staining utilizes the specificity of antibodies to an antigen that is expressed by a cell. A preliminary step in this kind of staining is to fix the cells and add a blocking reagent that will prevent the antibodies from binding to unspecific targets in the sample [56]. Fluorescent colours are attached to the antibodies that bind specifically to the specific antigens and the fluorescent dye ends up on a specific target within the cells. This was utilised to distinguish between leukocytes, cancer cells and platelets. The WBCs were stained with CD45 antibody which gave their membrane a red colour. The cancer cells were stained yellow with antibodies against keratin, and platelets were stained green with the CD61 antibody. All the cell nuclei were stained blue with DAPI.

Validation of IF staining was performed with a fluorescent microscope. Molecules have a unique spectre of wavelengths that they absorb. The microscope send out light with a short wavelength (often UV light) which excites the cells [57]. The most energy-rich light is absorbed by the fluorophores and the cells are looked at through different filters that absorb the other wavelengths, resulting in clear fluorescence of the sample against a dark background [57]. Fluorescence involves both excitation and emission, which are two bands of wavelengths. The excitation and emission are different for each fluorophore. Figure 6 below shows the excitation and emission of the fluorophores used for IF staining in this project. When staining with several fluorophores it is necessary that these do not overlap with each other. Specific filter combinations can favour the excitation and emission of particular fluorophores [57]. An overview over the filters used for microscopy of cells can be seen in Table 4.

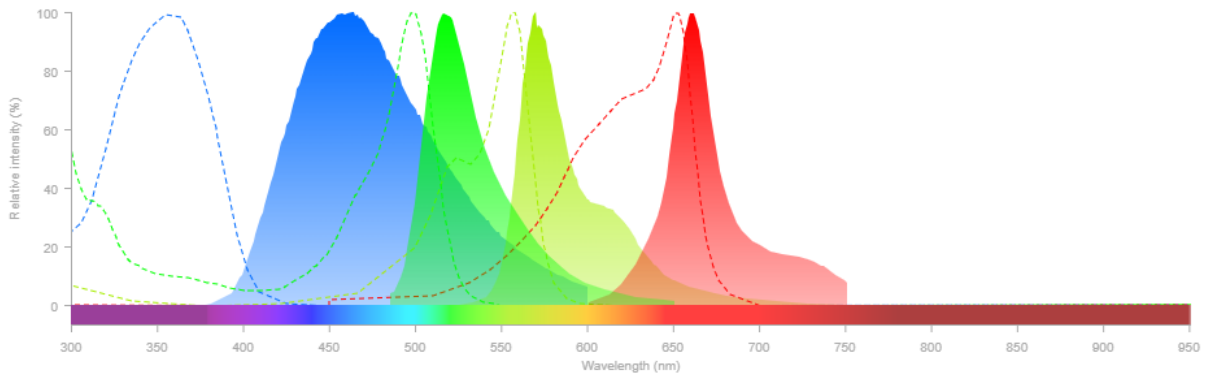


Figure 6. Excitation and emission of fluorophores. The diagram shows the excitation and emission intensities of the fluorophores DAPI (blue), FITC (green), eFluoro 570 (yellow), and APC (red). Dotted: excitation, filled in: emission. From ThermoFishers "Spectraviewer". [Fluorescence SpectraViewer (thermofisher.com)]

Table 4. Filter configurations for the Leica Microscope. The excitations and emissions in the different filter set of the microscope.

Visualised	Filter Set	Excitation	Emission
DAPI	DAPI	360/40	460/50
APC	Filter set 50 (APC)	640/30	690/50
Keratin	SpGold	547/12	572/22
Platelets	SpGreen	494/26	536/30

Microscopy was used to validate the experiments and to read the results. The slides were looked at with the eyes, as well as scanned in Metafer 4, RCDetect (V3.5.12). The exposure times used when scanning in Metafer are presented in Table 5. The focus parameters were set to FL20 and the scan was performed with a 10x objective. Pictures were taken in ISIS (V5.2.23), using the 10x and 40x objective for patient samples. The 100x objective was used during optimalisation if protocols. A scanning procedure for Metafer 4 can be found under Appendix C1 and the settings for the Classifier Setup can be found under Appendix C2.

Table 5. Exposure mode and integration time for the colours in Metafer. These parameters were used when scanning in Metafer. The parameters can be found under RCDetect – Classifier Setup.

Colour channel	Exposure mode	Integration time (seconds)
Blue	Fixed	0,0417
Green	Fixed	0,6250
Red	Fixed	4,7917

3 RESULTS

An overview of the experimental approaches applied in this study is shown in Figure 7. Optimalisation of immunofluorescence staining of leukocytes, cancer cells and platelets using antibodies were made prior to using the techniques on patient samples. Immunofluorescent staining was necessary to separate between the different cells that were isolated during enrichment. Validation of enrichment was also done before applying the method to patient samples. The results presented in this section are divided into three parts – optimalisation, validation and patient samples.

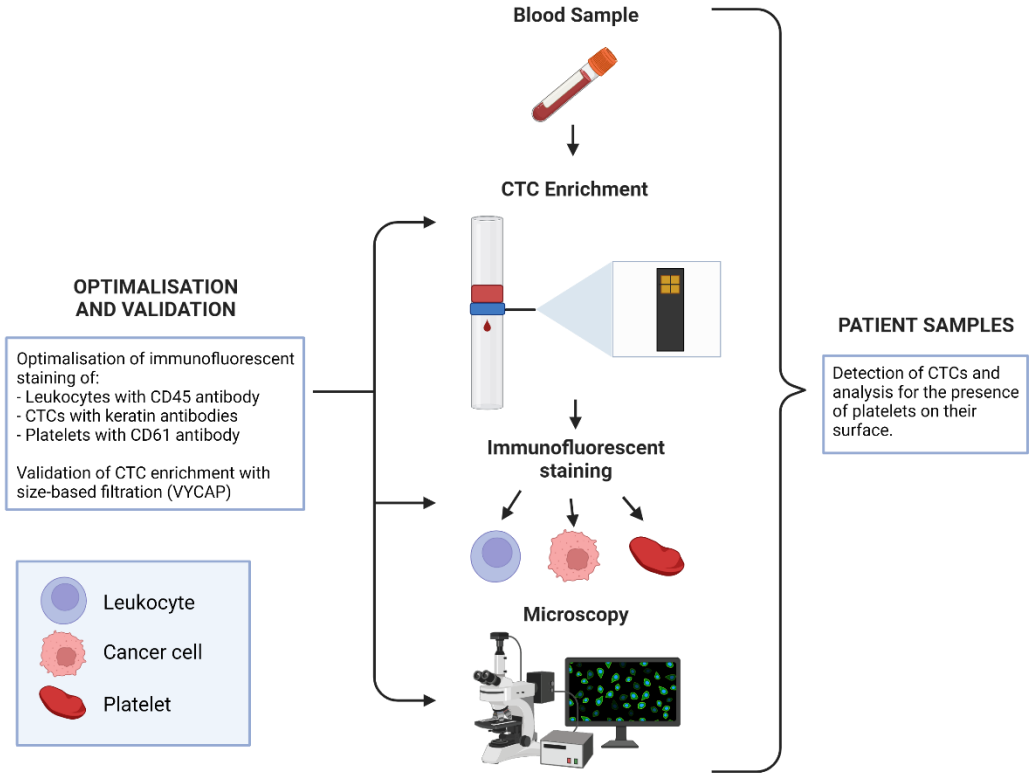


Figure 7. Workflow. The main flow diagram (with pictures) is what was ultimately used for patient samples. The individual parts of the diagram were optimised and validated prior to being used in experiments with patient samples. Figure adapted from "Split Workflow (Layout)", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

3.1 Optimalisation of Immunofluorescence protocols

3.1.1 Immunological staining of cancer cells and PBMC

The purpose of this experiment was to validate staining protocols, fluorophores, and fluorescence filters as well as establishing suitable exposure times for taking pictures with fluorescent microscopy.

White blood cells and ZR75-1 cells were successfully isolated using protocols IV and V. The cells were divided into 4 microtubes and stained according to table 6 below. Only the cells in microtube number one were stained with all antibodies as described in the staining protocol.

Table 6. Antibody concentrations for immunofluorescent staining of cancer cells and PBMC. Following are the concentrations of DAPI and the antibodies against keratin (AE1/AE3) and CD45. DAPI stains the nuclei of the cells. Keratin antibodies stain the cancer cells and CD45 antibody stain the membrane of the leukocytes. Concentrations are given as dilution factors.

Microtube	Concentration DAPI (0.1 mg/ml)	Concentration Anti-Human Pan cytokeratin AE1/AE3	Concentration Anti-CD45 APC
1	1:100	1:50	1:50
2	1:100	-	-
3	-	1:50	-
4	-	-	1:50

The stained cells were transferred onto 4 separate slides and looked at under a microscope. Suiting filters for the fluorophores DAPI, eFluor 570 and APC were found to be DAPI, SpGold and Filter set 50 (APC) (for more details, see Table 4 in Section 2.2.5). The slides were used to test different exposure times to find what gave appropriate luminosity. The optimal exposure times are presented in Table 7 under fixed integration times. By studying object-glasses 2-4, where the cells were stained with one antibody, it was determined that there was no cross-signal from the different channels. The immunofluorescent staining of the ZR75-1 cell line was specific and suitable for distinguishing between the cancer cells and PBMC. The staining of leukocytes with CD45 antibody appeared to be bright and distinct, specifically concentrated to the membrane of the cell (see Figure 8). The experiment was successful except from the samples containing few cells.

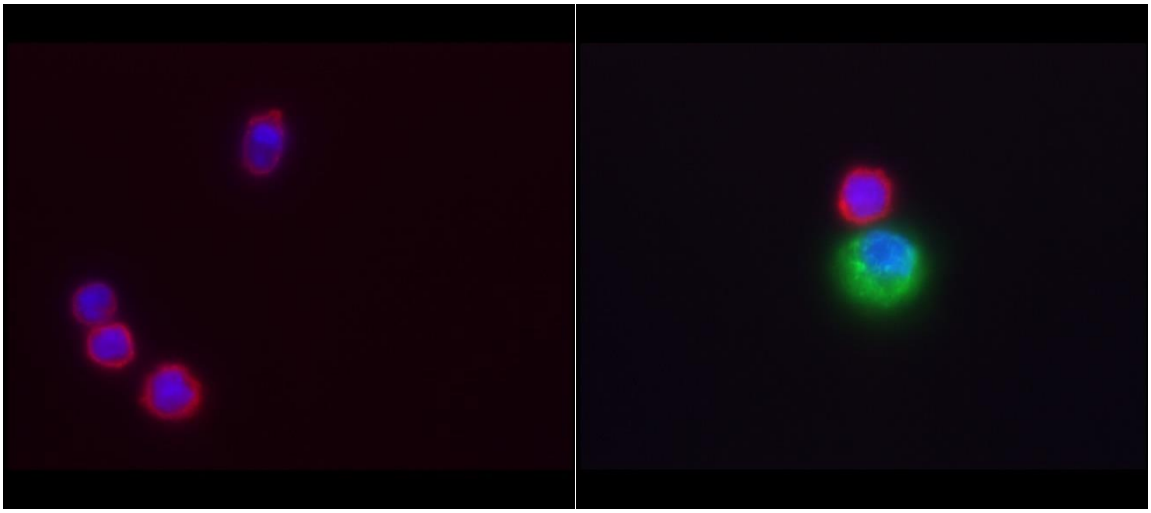


Figure 8. Immunofluorescent staining of leukocytes and ZR75-1 cells. The membrane of the leukocytes are stained red with antibodies against CD45 (left and right). The ZR75-1 cells are stained green with keratin antibody (right). The nuclei of the cells are stained with DAPI (blue). The difference in size between the leukocytes and the ZR75-1 cells is notable (right). The pictures were taken in the ISIS software using the 100x objective.

Table 7. Fixed integration times. The following integration times were found to work best for immunofluorescent staining of cancer cells, PBMC and platelets.

Visualised	Filter Set	Fixed integration times (s)
DAPI	DAPI	0.08
APC	Filter set 50 (APC)	3.00
Keratin	SpGold	1.00
Platelets	SpGreen	0.50

3.1.2 Staining of cancer cells and platelets with antibodies against keratin and CD61

The last experiment utilised the pan cytokeratin AE1/AE3 antibody for staining of cancer cells. The purpose of this experiment was to test out another keratin antibody (C11) and the combination of the two in order to determine the most favourable staining of ZR75-1 cells. It was assumed that optimal staining of ZR75-1 should be applicable to staining of CTCs. Another aim of the experiment was to titrate the concentration of CD61 antibody to determine which concentration would be optimal for staining of platelets. In steps 2 and 3 of protocol VI, 100,000 leukocytes and 50,000 ZR75-1 were transferred to each of 8 microtubes.

In step 6 of protocol VI, antibodies were added to the 8 microtubes after Table 8 below. Tubes 1-4 were used to find the optimal concentration of the CD61 antibody. Tube 5 was used to test the unspecific binding of CD61 antibody to the cells and/or cross-signal from the CD61 antibody in the SpGold filter set. Tubes 6-8 were used to test the two keratin antibodies separately and the combination of them.

Table 8. Added antibodies for staining of cancer cells, leukocytes, and platelets. The table shows the concentration of antibodies added to each of the microtubes in protocol VI. The antibodies were added for staining of cancer cells with the keratin antibodies AE1/AE3 and C11, staining of leukocytes with CD45 antibody, and for staining of platelets with CD61 antibody. Concentrations are given as a dilution factor. Tube 1-4 was used to find the optimal concentration of CD61 antibody for staining of platelets. Microtube number 5 was used to test for non-specific binding of the CD61 antibody. Tubes 6-8 were used to test the keratin antibodies separately and in combination.

Microtube	Concentration CD45 ab	Concentration AE1/AE3 keratin ab	Concentration C11 keratin ab	Concentration CD61 ab
1	1:50	1:100	-	1:200
2	1:50	1:100	-	1:100
3	1:50	1:100	-	1:50
4	1:50	1:100	-	1:25
5	-	-	-	1:50
6	1:50	1:100	-	-
7	1:50	-	1:100	-
8	1:50	1:100	1:100	-

The CD45 staining of leukocytes was bright and distinct and there was no keratin staining of the white blood cells. The CD45 antibody bounded specifically to the membrane of the cells (see Figure 9 below). Some leukocytes were observed to have less CD45 colour than others. The experiment gave an indication as to how the cells looked when stained with keratin antibodies (see Figure 10). It was determined to use the combination of the two keratin antibodies in further experiments. For additional pictures from this experiment, see Appendix D1.

Unfortunately, the number of platelets co-isolated in this experiment was too low to draw conclusions regarding the optimal antibody concentration. However, we observed only some CD61 staining on PBMCs or ZR75-1 cells even with the highest antibody concentration tested.

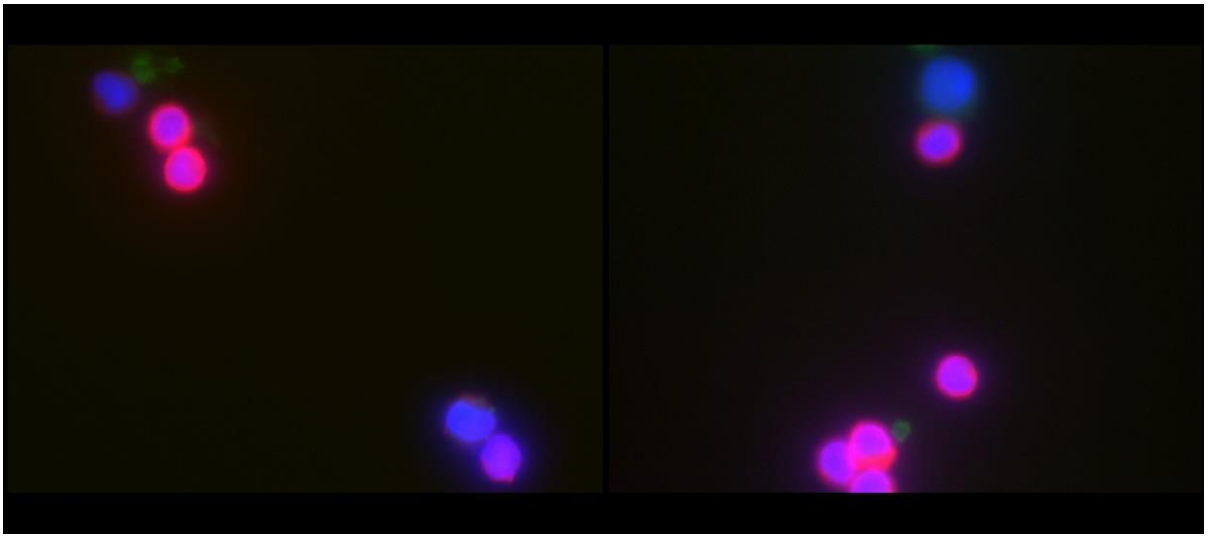


Figure 9. Immunofluorescent staining of leukocytes and platelets. WBCs and platelets are stained with antibodies against CD45 (red), keratins (yellow), and CD61 (green). The nuclei of the cells are stained with DAPI (blue). The pictures were taken with the ISIS software, using the 100x objective. The area of the pictures is quite small; therefore, no cancer cells are observed in the pictures. These pictures are from microtube number 4.

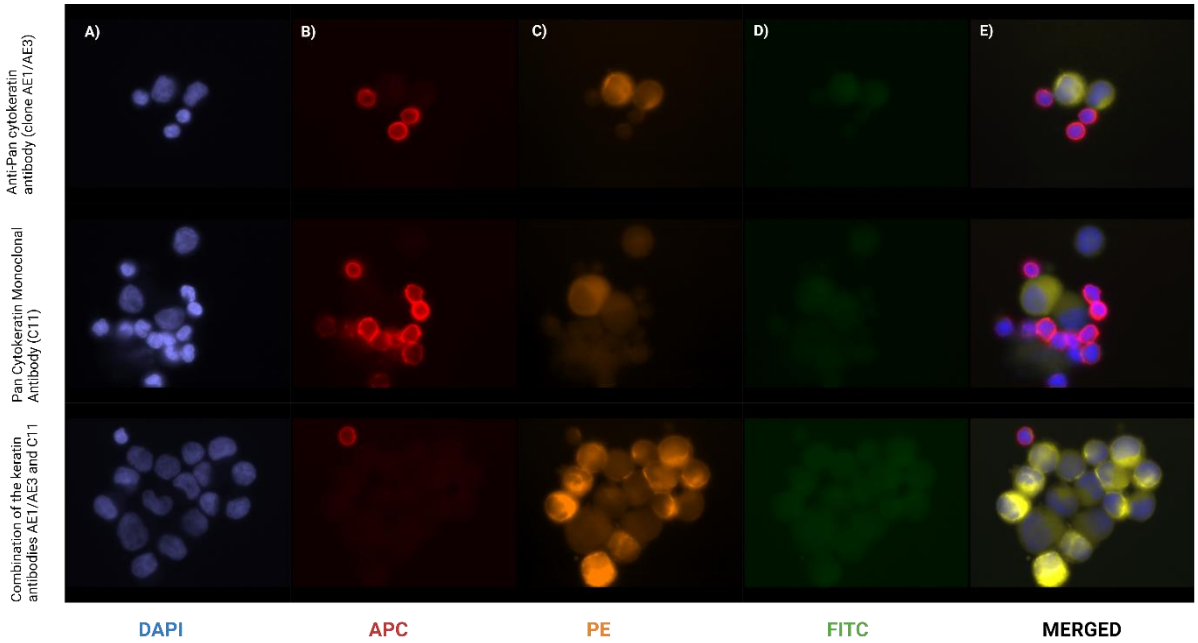


Figure 10. Immunofluorescent staining of cancer cells, leukocytes, and platelets. The cells are stained with antibodies against CD45 (red) and keratins (yellow). The nuclei of the cells are stained with DAPI (blue). From top to bottom: tubes 6-8 in the experiment for staining of cancer cells with different antibodies against keratin. All the samples were stained with CD45 (1:50). The first sample was stained using AE1/AE3 antibody. The second sample was stained with C11 antibody. The third contained both antibodies against keratin. There is however almost no cross-signal from the keratins in the APC channel. The green colour in the FITC channel suggests some cross-staining of the keratins to the FITC channel. The pictures are taken in the ISIS software, using the 100x objective.

3.1.3 Isolation of platelets

To increase the number of platelets in the CD61 antibody titration experiment, we specifically isolated platelets using protocol VII. Isolation, fixation and staining of platelets was done several times in order to titrate the volume of CD61 antibody to decide the optimal amount to use for patient samples. The first and second time the experiment was conducted, no platelets could be found when looking in the microscope. Somehow, the platelets were lost, or they aggregated. In a third experiment, an additional microtube was used to be able to check after each step in the protocol if there were any platelets left and if they got activated and aggregated. It was determined that the main cause was that the platelets got lost, not that they aggregated. It required the removal of one washing step to recover the platelets. During a fourth experiment, using protocol VII, two parallels with microtubes were used in order to learn if the procedure worked best with fixation or without fixation. It was observed that the platelets that were fixed aggregated less than the platelets that were not fixed. Therefore, in the final protocol presented under methods (protocol VII) the platelets are fixed. Protocol VII found under methods (section 2.2.3) is the latest version that was adapted to work best.

3.1.4 CD61 antibody staining optimisation

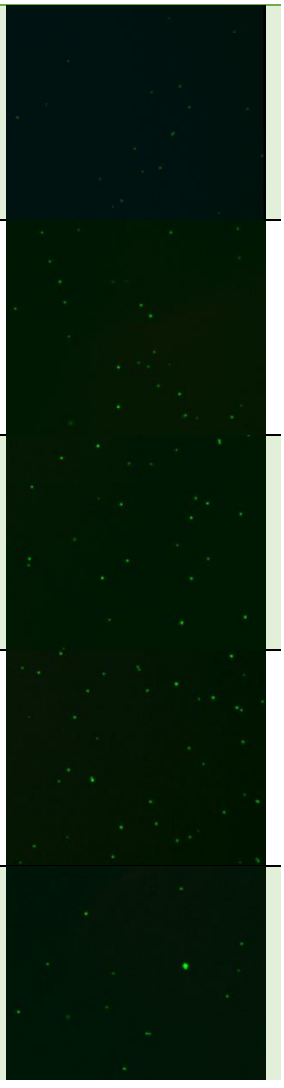
For titration of CD61 antibody, a platelet suspension isolated from normal plasma using protocol VII was divided into 5 Eppendorf tubes with 15 µl in each in step 7 of protocol VII. In step 9, antibodies were added according to the Table 9 below. Tubes 1-4 were used to find the optimal concentration of the CD61 antibody, tube 5 was used to test cross-signal from the CD61 antibody in the SpGold filter set.

Table 9. CD61 antibody titration. Concentrations of CD61 antibody added to each of five microtubes with platelets for titration of the antibody. The experiment was done to determine the optimal amount of CD61 for immunofluorescent staining of platelets.

Microtube	Concentration CD45 ab	Concentration AE1/AE3 keratin ab	Concentration CD61 ab
1	1:50	1:100	1:200
2	1:50	1:100	1:100
3	1:50	1:100	1:50
4	1:50	1:100	1:25
5	-	-	1:50

The 5 mixes of platelets and CD61 antibody were transferred to separate object glasses and observed in the microscope. Pictures were taken in the ISIS software with fixed exposure time, to be able to compare the intensity of the staining. The intensity of the staining was quite equal for all the microtubes, except from when using the antibody concentration of 1:200, which gave weaker signals. See table 9 below. Taking the staining of PBMC and ZR75-1 cells from section 3.1.2 into account, it was concluded that a 1:50 concentration of the CD61 antibody was optimal for platelet staining in samples consisting of both leukocytes, tumour cells and platelets.

Table 10. Titration with CD61 antibody for staining of platelets. Different concentrations of the CD61 antibody was added to 5 separate microtubes in order to determine the optimal concentration for immunofluorescent staining of platelets. The following table shows pictures of how the staining looked for each of the microtubes. The pictures were taken in the ISIS software using the 10x objective and fixed exposure time. These pictures are cut-outs from a larger area and the intensity has been adjusted up for a clearer view of the slides.

Microtube	*Concentration CD45 ab*	*Concentration AE1/AE3 keratin ab*	*Concentration CD61 ab*	*Microscopy picture*
1	1:50	1:100	1:200	
2	1:50	1:100	1:100	
3	1:50	1:100	1:50	
4	1:50	1:100	1:25	
5	-	-	1:50	

3.2 Validation of VYCAP size-based CTC enrichment

We validated the VYCAP size-based CTC enrichment procedure by analysing blood samples from healthy volunteers with a known number of ZR75-1 cell line cells spikes in. Three blood test tubes were attained from a healthy donor. A volume corresponding to 1000 ZR75-1 cells was added to two of the blood samples, after which the tubes were inverted 3x. The third tube was a negative control and did not contain any spiked-in cancer cells. The experiment was done multiple times, first with EDTA tubes, and subsequently with CellSave blood collection tubes, which contains a cell fixative. When spiking into an EDTA tube, it is favourable to proceed directly to filtration. When spiking was done into CellSave tubes, the tube was set to rest for one hour before proceeding to filtration. After spiking of blood samples, protocol VIII was followed for enrichment of the spiked-in cancer cells.

At first, the experiments were performed with fresh EDTA blood. There was however quite extensive observable damage to the cells. The cells were smudged out over the filter and their membrane was smashed out, in addition to the cells having malformed nuclei (for a view of damaged cells, see Appendix D2). The lack of intact cells made the morphologic evaluation effortful. An attempt to solve the problem by lowering the pressure and stopping the pump earlier was made, which resulted in slightly less damaged cells. Since the problem could not be solved for EDTA blood, the decision was made to switch to CellSave tubes. For a view of how cells looked after filtration with CellSave blood, see Appendix D3). Table 11 below presents the number of recovered cells from each of the validation experiments. The average recovery percentage was 43,4% for EDTA blood and 48,7% for CellSave blood.

Table 11. Overview of validation experiments. 1000 ZR75-1 cells were spiked into two blood sample parallels for EDTA-tubes respectively CellSave tubes. The table shows the number of recovered ZR75-1 cells after filtration. The cells were counted with fluorescence microscopy.

Parallel	Recovered ZR75-1 cells in EDTA-tubes (out of 1000 cells)	Recovery percentage ZR75-1 cells in EDTA-tubes (%)	Recovered ZR75-1 cells in CellSave-tubes (out of 1000 cells)	Recovery percentage of ZR75-1 cells in CellSave tubes (%)
1	389	38,9	428	42,8
2	478	47,8	546	54,6
Average recovery	434	43,4	487	48,7

During the validation procedures it was also observed that some cells were weakly positive for both CD45 and keratin. These cells were observed in the spiked samples as well as in the negative control samples. Although the presence of these cells in the negative control indicates that these cells are present in blood before spiking, an additional experiment was made to determine if ZR75-1 could give signal in both the keratin and the APC channel (see Section 3.2.1 below).

3.2.1 Filtration of ZR75-1 cells

There were two reasons to filtrate solely ZR75-1 cells. Reason number one was to investigate how the cells tolerate filtration, as there was observed a lot of damaged and smashed cells during the validation experiments. The other reason for filtration of ZR57-1 cells was to observe how the staining of these cells looked in Metafer and in the microscope and to determine if there was any cross-signal from the cell-line in the APC channel.

A volume corresponding to 10 000 ZR75-1 cells were mixed in a tube with 10 ml of VYCAP buffer (PBS/1% BSA) before transferring the mix to the pump unit and starting filtration with the pressure set

to 15 mbar (see Protocol IX under Appendix C1 for more details). This means that the ZR75-1 cells were not fixed before filtration and the outcome comparable to using EDTA blood collection tubes. The recovery of the cells was not counted.

The filtered cells were stained with DAPI, keratin antibodies, antibody against CD45, and CD61 antibody as described in Protocol VIII. It was concluded that the ZR75-1 cells did not tolerate filtration well without a fixation step. Even though the pressure was lowered to 15 mbar, the cells were observed to be quite damaged (see Figure 11). This supported our choice to use CellSave tubes instead of EDTA blood collection tubes. Besides, there was no signal from the ZR75-1 cells in the APC channel or the PE channel (used for CD45 and CD61 staining), emphasizing the specificity of the CD45 and CD61 antibodies.

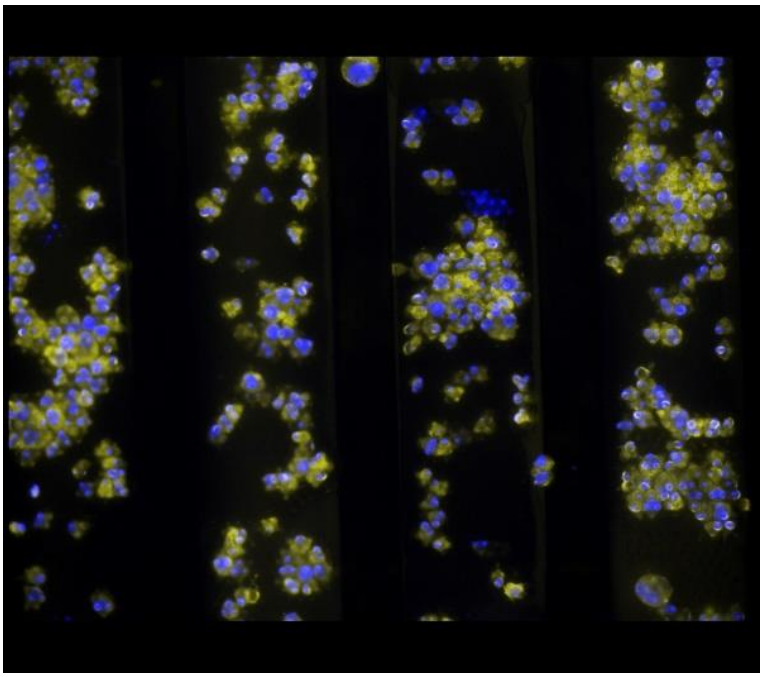


Figure 11. Immunofluorescent staining of ZR75-1 cells after filtration with VYCAP. The cell line is stained with antibodies against keratins (yellow), CD45 (red), and CD61 (green). The nuclei of the cells are stained with DAPI (blue). As noticeable, there is no red or green colours due to the lack of leukocytes and platelets in the sample, which consisted entirely of ZR75-1 cells. Extensive damage to the cells can be observed after filtration. The picture was taken with the ISIS software, using the 10x objective.

3.3 PATIENT SAMPLES

There were in total 10 patient samples analysed, from 7 individuals with metastatic breast cancer. The patients are a part of the MBC-study, mentioned under Section 2.1.4. The section about patient samples is divided into three sections – CTCs detected in patient samples, platelets detected on CTCs, and CTC clusters.

Table 12 provides an overview of the details and results from all patient samples. The cells that have been detected as CTCs were keratin positive (KRT+) and negative for CD45 (CD45-). Potential CTCs is a category containing cells that were both KRT+ and CD45+. The requirement for CTC-platelet interaction was that the platelets were attached to the CTC, on top of it, adjacent to it or nearby the CTC. The threshold distance for a platelet-CTC interaction was decided to be ≤ 0,3 µm.

The scanning of patient samples revealed a great deal of staining of the filter in the microsieve (see Appendix D5). The filtration pressure and time varied between patient samples. For details of each of the samples see Appendix D6.

*Table 12. Patient samples. Blood samples were collected from 7 individuals. For three of the patients (A, B and C) blood was taken at two different time points, the occasion indicated with lowers numbers. Blood samples were drawn in either EDTA blood collection tubes or CellSave blood collection tubes. Fresh EDTA blood was filtrated the day of the blood draw while the number of days from blood draw to filtration varied between 0-2 days for the samples in CellSave tubes. Abbreviations: CTC-S: single CTC, CTC-C: CTC cluster. * These observations are potential CTC-clusters, the cells in the cluster were positive for both CD45 and keratin.*

Blood Sample Number	Blood Collection Tube	Number of days from blood draw to filtration	Volume of sample	CTC-S	Number of CTC-S with platelets	Potential CTC-S	Number of CTC-C	Number of CTC-C with platelets
A_1	EDTA	0	4,5 ml	35	2	95	0	-
A_2	CellSave	2	5 ml	24	6	179	0	-
B_1	EDTA	0	4,5 ml	8	0	56	4	4
B_2	CellSave	2	5 ml	5	0	13	0	-
C_1	EDTA	0	4,0 ml	1	0	22	0	-
C_2	CellSave	1	5 ml	23	9	94	3	3
D	CellSave	0	10 ml	0	-	6	0	-
E	CellSave	0	10 ml	10	7	20	1*	1*
F	CellSave	2	5 ml	27	11	94	0	-
G	CellSave	1	5 ml	8	1	44	1*	1*

3.3.1 CTCs detected in patient samples

CTCs could be detected in 9 out of 10 patient samples (90%) using VYCAP size-based filtration technique. In the two samples from the same patient, the number of CTCs was similar – which indicates replicability of the filtration technique. Many immune cells are placed in the category of possible CTCs, since they were positive for both CD45 and keratin. They can be distinguished by being polymorphonuclear, meaning the nucleus is divided in several parts. Figure 12 shows two CTCs and what is likely an immune cell that is keratin positive. Several nuclei are clearly visible within the cell.

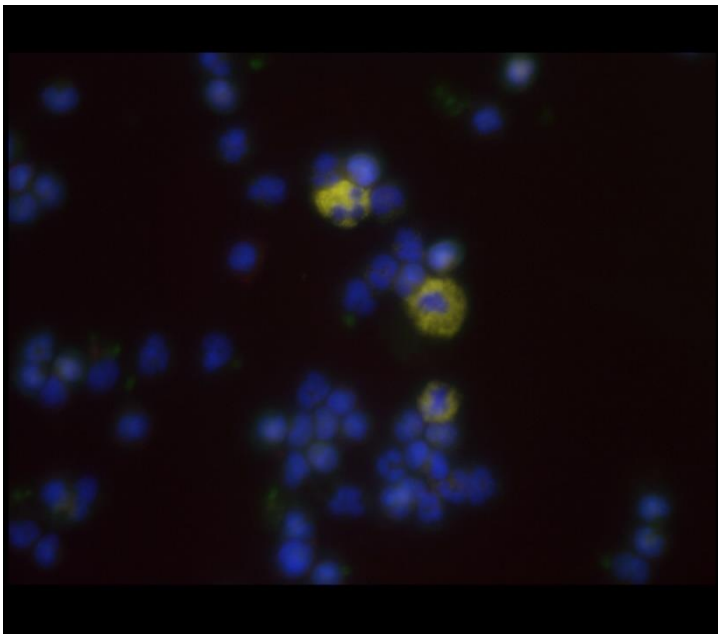


Figure 12. Two CTCs and one possible CTC. The picture is showing immunofluorescent staining of leukocytes, CTCs, and platelets with antibodies against CD45 (red), keratins (yellow), and CD61 (green). The nuclei of the cells are stained with DAPI (blue). The picture shows two CTCs (lower) and one possible CTC (upper). The possible CTC is KRT+ but is most likely an immune cell due to the polymorphonuclear appearance. The picture was taken with the ISIS software, using the 40x objective.

3.3.2 Platelets detected on CTCs

Platelets were observed in 67% of the cases where detectable CTCs were found (in 6/9 patients). The number of CTCs that interacted with platelets varied greatly between patients, as can be seen in table 12 and figure 13. The average percentage of CTCs with platelets in the six patients that had detectable platelet-CTC interaction was 32,2%. For the percentages of individual patients, see Appendix D4. The percentage of CTC with platelets in total for all patient samples was:

$\frac{36}{141} \times 100\% \approx 25{,}5\%$

Figures 14 and 15 show platelets on circulating tumour cells and figure 16 shows platelets on all 3 possible CTCs in the picture. Some samples had unspecific binding of the CD61 antibody.

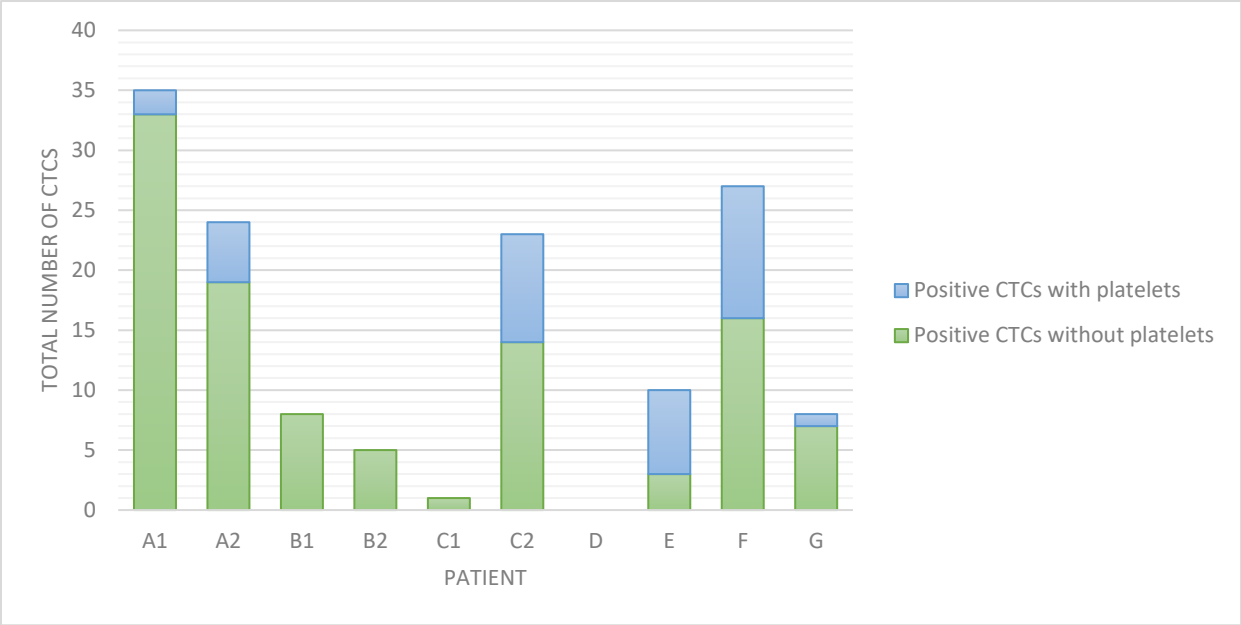


Figure 13. Patient samples diagram. The diagram exhibits the total number of CTCs and the distribution of CTCs with and without platelets in the patient samples.

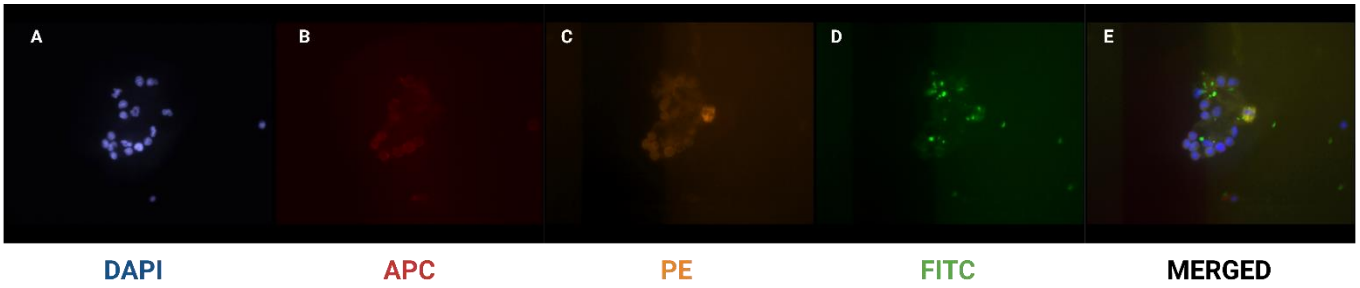


Figure 14. Platelet Interaction with Circulating Tumour Cell. A) The nuclei of the cells are stained blue with DAPI. B) Immunofluorescent staining of leukocytes with an antibody against CD45 (red) is seen in the APC channel. C) Immunofluorescent staining of CTCs with antibodies against keratin is seen in the PE channel. This picture contains one CTC. D) Immunofluorescent staining of platelets with an antibody against CD61 is observed in the FITC channel. E) Merged colours. Notice the two platelets attached to the circulating tumour cell. The picture was taken with the ISIS software, using the 40x objective. The figure was created with BioRender.com.

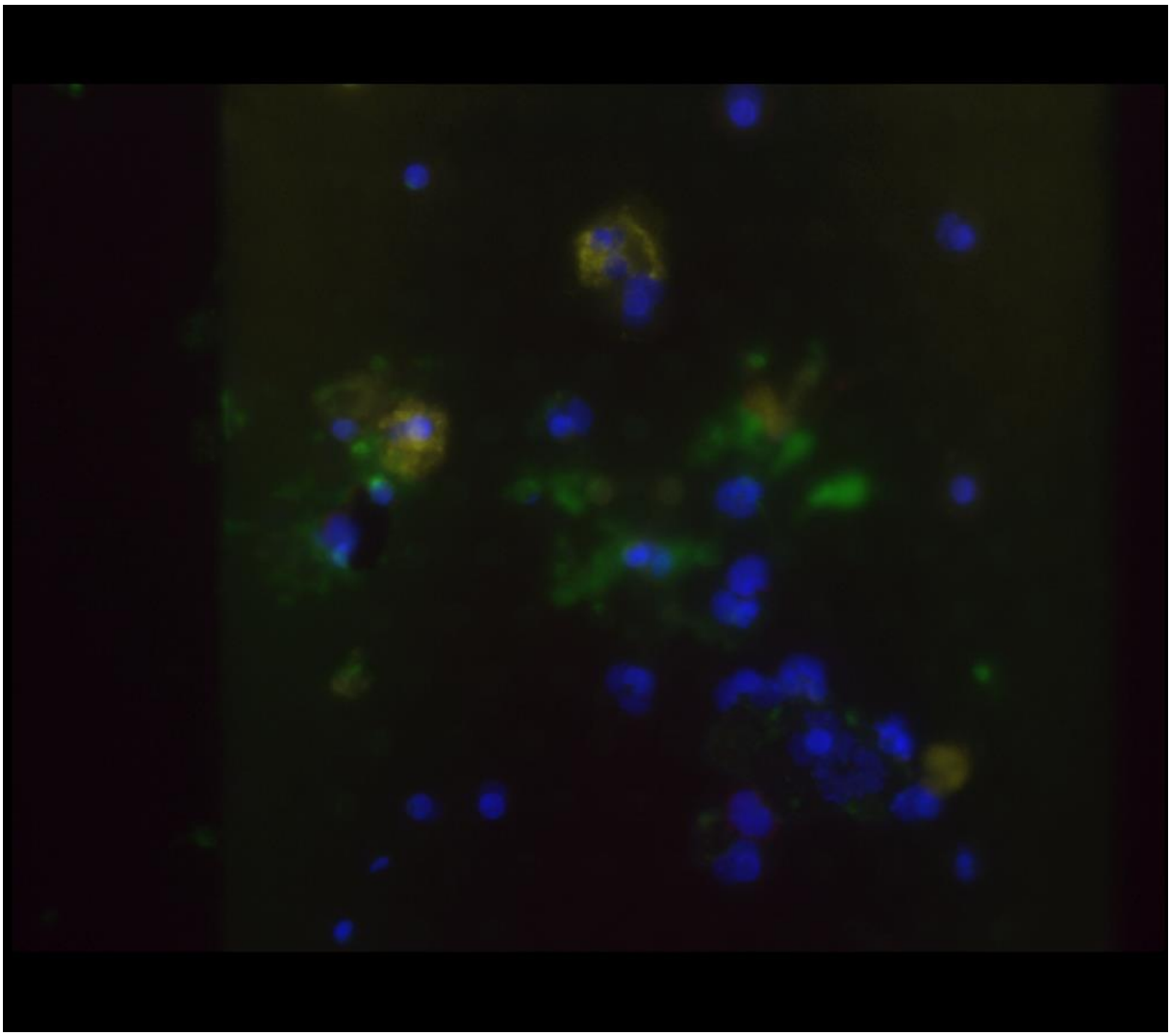


Figure 15. Platelet Interaction with Circulating Tumour Cell. Immunofluorescent staining of leukocytes, CTCs, and platelets with antibodies against CD45 (red), keratins (yellow), and CD61 (green). The nuclei of the cells are stained with DAPI (blue). The picture shows a CTC with two platelets interacting. The picture was taken in ISIS software using the 40x objective.

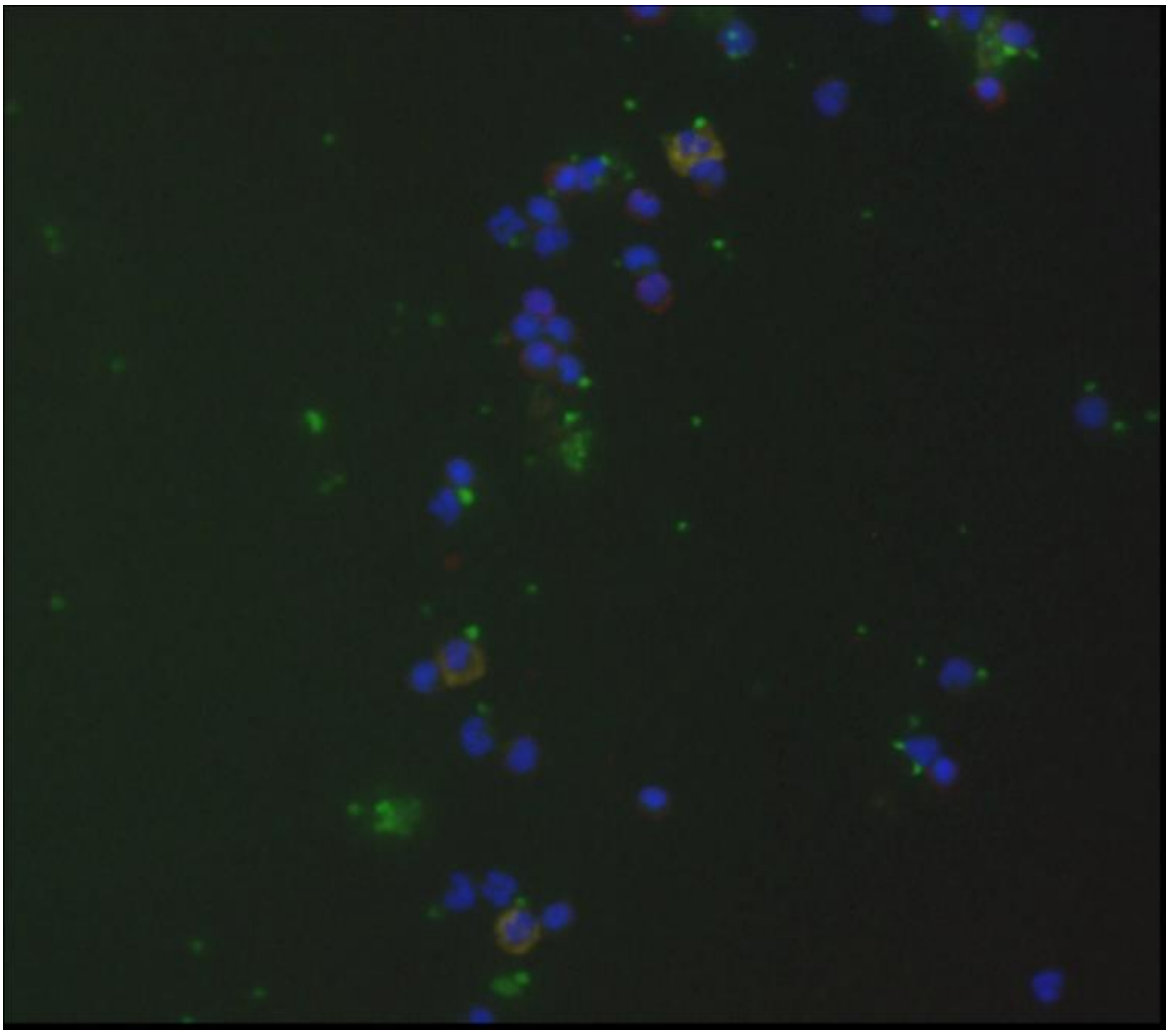


Figure 16. Platelet Interaction with possible CTCs. Immunofluorescent staining of leukocytes, CTCs, and platelets with antibodies against CD45 (red), keratins (yellow), and CD61 (green). The nuclei of the cells are stained with DAPI (blue). These three possible CTCs interacted with at least 1 platelet each. The picture was taken with the ISIS software, using the 10x objective and the zoom function.

3.3.3 CTC-clusters

CTC clusters were found in 2 patient samples, and two additional patient samples were found to have something that resembles CTC clusters but the cells in the cluster were both KRT+ and CD45+. All the clusters observed in this project had platelets within them. For examples of CTC cluster, see Figures 17 and 18.

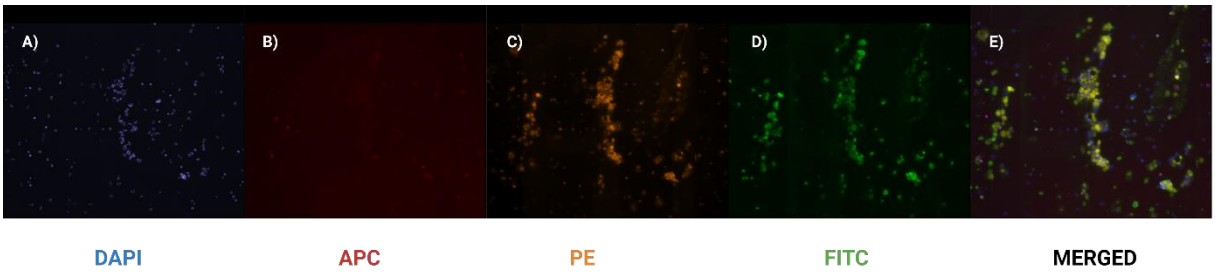


Figure 17. CTC clusters. A) DAPI staining of cell nuclei. B) APC channel showing leukocytes stained with CD45 antibody. As can be seen, there are few leukocytes present. C) Staining of CTCs with keratin antibodies is seen in the PE channel (orange). D) Staining of platelets with CD61 antibody is seen in the FITC channel. A lot of unspecific binding of the CD61 antibody was observed in this sample. E) Merged colours.

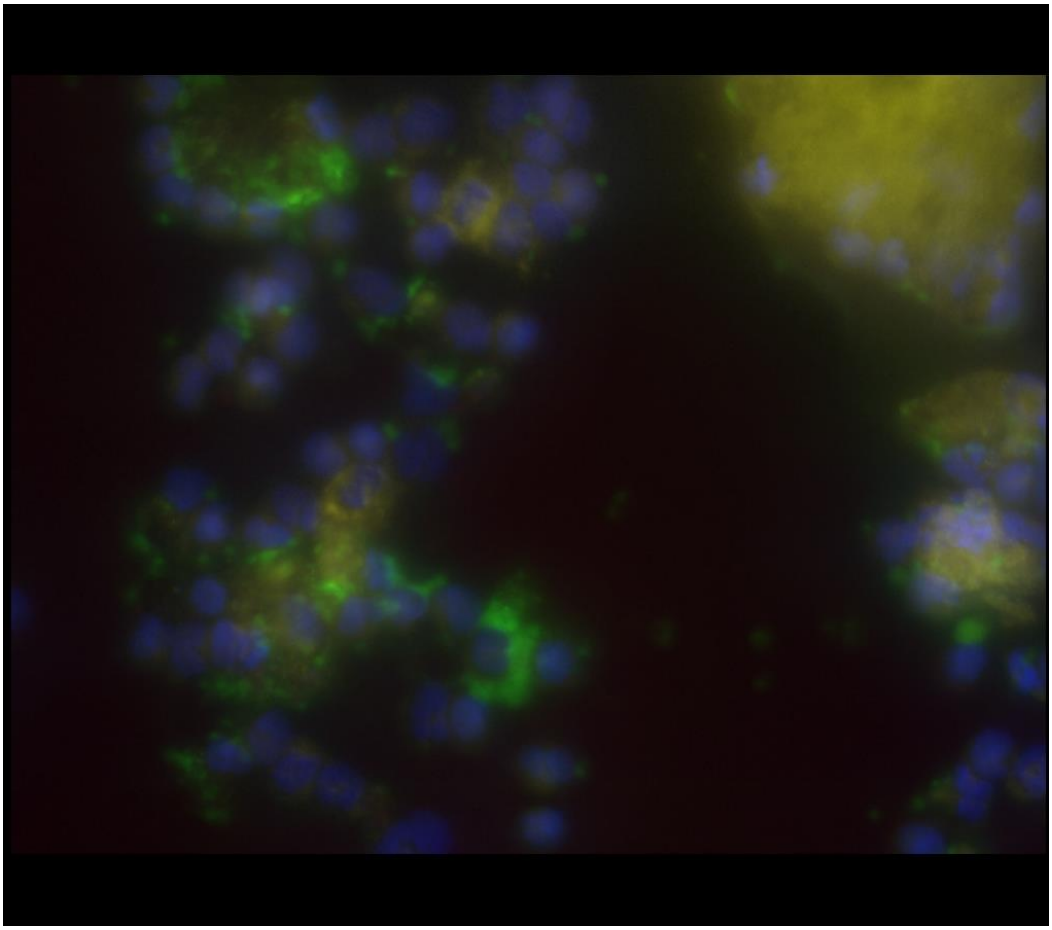


Figure 18. CTC cluster. Immunofluorescent staining of leukocytes, CTCs, and platelets with antibodies against CD45 (red), keratins (yellow), and CD61 (green). The nuclei of the cells are stained with DAPI (blue). Platelets are clearly observable within the cluster. The picture was taken in the ISIS software using the 40x objective.

4 DISCUSSION

4.1 CHALLENGES IN CTC ENRICHMENT AND DETECTION

The biggest challenge in CTC enumeration is the fact that CTCs are rare cells, and even after isolation of CTCs there are still a lot of leukocytes left. It can be challenging to separate the cancerous cells from the many cell types found in normal blood as some of the other cells exhibit the same or similar molecular markers as the CTCs. For example; some immune cells exhibit a positive marker for both CD45 and keratin, posing a challenge in distinguishing between them.

Methods based on antigens are insensitive to the sizes of CTCs, but the techniques do not manage to detect cancer cells with lower expression of the target antigens [58]. Positive enrichment using EpCAM presents with certain challenges. It is known that EpCAM is not a universal marker that functions for detection of all CTCs. In cancers with low or variable EpCAM expression, or with CTCs that have gone through EMT, the CellSearch system together with other EpCAM-based CTC enrichment technologies will probably be ineffective [23, 51].

Another challenge is the short lifetime of CTCs in the blood. They are degraded due to physical stress or eliminated through anoikis. It is no coincidence that the lifetime of blood cells is greatly lower than those of most other cell types. On average, a RBC lives for about 115 days [59]. Varying on the type of leukocyte, the lifetime can span from a few hours up to years, but on average they live for 13-20 days [60, 35]. The fact that blood cells have shorter lifetimes can be explained in part by the mechanical stress that these cells are put through when circulating through the body. CTCs do not tolerate this stress as well as the blood cells and thus are broken down within hours. Only a small fraction of CTCs survive and can interact with other cells such as neutrophils, macrophages, MDSCs and CAFs. Recent evidence suggests that interaction between CTCs and blood microenvironment is necessary for CTC adhesion to epithelial cells and thus to invasion and metastasis [21].

4.2 ENRICHMENT OF CTCS WITH SIZE-BASED FILTRATION

4.2.1 Choice of CTC enrichment method

As seen in section 1.2.6, there are many ways to enrich CTCs from whole blood. There are advantages and disadvantages with all methods, also with regard to potential interactions between CTCs and platelets.

Platelets binding to CTCs may interfere with negative depletion enrichment of CTCs if the antibodies used for depletion are also binding to platelets. For instance, the MINDEC negative depletion method was developed for maximal depletion of PBMCs through antibodies targeting many blood cell types [17]. If the platelets bound to CTCs exhibit some of the same markers as the leukocytes, it may result in the platelet-covered CTCs being selected away and only the CTCs without platelets being detected. On the other hand, a positive enrichment such as the CellSearch system using EpCAM for selecting CTCs may also be a problem for finding CTCs with platelets. CTCs are selected based on the presence of EpCAM on their surface [61, 62]. If the CTCs are covered with platelets, it is possible that the antibodies cannot attach to their receptors and the platelet-covered CTCs will go undetected.

Considering the mechanisms of enrichment from biological properties in relation to how platelets and CTCs are believed to interact, it was suggested to enrich CTCs based on physical properties instead.

The benefit of enrichment based on physical properties would be that the method does not influence the interaction between CTCs and platelets as much as the methods based on biological properties. Size-based filtration is independent of antigen expression, and it could therefore be more suitable for detection of platelets on CTCs. The hope was that the use of size-based filtration would enable us to see the interaction between platelets and CTCs more clearly, and possibly result in a higher number of detected CTCs with platelets attached.

The advantages of size-based filtration is the rapid processing of large volumes and it's high efficiency. The procedure is easy to follow, and it does not require much time compared to other methods that are often quite time-consuming [51]. There are however obvious limitations with size-based enrichment technologies. The CellSearch system has identified CTCs of various sizes, posing a challenge for methods utilising filtration as there is a risk of losing CTCs that are smaller than the pores of the filter [52, 58]. Other disadvantages are membrane clogging and low purity. Microfiltration also presents with the difficulty to detach CTCs from the filter [51]. CTCs are thought to be more rigid and less deformable than other hematopoietic cells [52]. However, due to the fact that some CTCs undergo EMT, it is also likely that they can exhibit the same deformability as leukocytes and therefore pass the filter more easily [52]. It is possible to imagine that filtration is more suitable for the detection of CTC-clusters than that of single CTCs.

4.2.2 CTC enrichment by VYCAP microsieve technology

The recovery of ZR75-1 cells from the validation experiments were quite low, with an average percentage of 43,4% for EDTA blood and 48,7% for CellSave blood. A low recovery may be attributed to smaller cell size and larger pores, as cells that are smaller than the pores can go through the filter. VYCAP's pore size of 5 µm may be too wide for the cells. On the other hand, in an article by Frank A.W et al. [58] it is stated that they found the 5 µm microsieve to have the best performance when comparing filtration devices. They found the microsieve to have > 80% recovery for the cell-lines MDA-231, SKBR-3 and PC3-9 [58]. This raises the question of whether some cell lines are more suitable for filtration than others. The low recovery of ZR75-1 cells in the validation experiments is most likely dependent on the size and deformability of the cell line. It is possible that ZR75-1 cells are smaller than the other cell lines used by Frank A.W et al., or that they are more deformable and thus can pass through the filter more easily. The observation from microscopy of ZR75-1 cells imply that these are fairly large cells, so the low recovery of the validation experiments is likely dependent on other factors than cell size. Other size-based systems such as the Siemens size-based filtration have reported higher average recoveries. The Siemens filtration had an average recovery percentage of 56% for the NCI-H1563 cell line compared to VYCAP's average recovery percentage of 32% for the same cell line [22]. However, there are also reports of high CTC recoveries above 70% with the VYCAP technology [55]. This is in large contrast to the results of the validation experiments from this project, where the best recovery average was < 50%. This project has found size-based filtration with VYCAP equipment to have a lower recovery than that of other enrichment techniques, such as immunomagnetic depletion by the MINDEC method [63]. In the previously mentioned article by Frank A.W. et al. they evaluated different filters for size-based enrichment of CTCs, looking at the material of the filter, pore spacing, thickness, area, number of pores, pore size, and porosity. They describe the ideal filter for CTC enumeration as follows: *"The ideal filter for CTC enrichment is constructed of a stiff, flat material, is inert to blood cells, has at least 100,000 regularly spaced 5 µm pores for 1 ml of blood with a ≤ 10% porosity."* [58]

Containing 160,000 precise pores with a diameter of 5 µm and being constructed by a stiff material, the VYCAP microsieve meet some of these requirements. There is a limited number of pores on the microsieve. This also influences the recovery. It has been shown that CTC recovery is constant until

around 2% of pores in the filter membrane are occupied [58]. The volume of the samples will probably affect the outcome of the filtration. The larger the sample volume, the higher number of pores will get occupied by larger cells which are not necessarily CTCs. In the future, additional validation experiments should be performed using other cell lines to get a better understanding of the recovery rate using VYCAP.

Another limitation with size-based filtration is the pressure. VYCAP´s website states that:

"The silicone nitride (SiNi) filter membrane has a thickness of 1 µm which is supported by a structure below the membrane. Because of this very small thickness, the required pressure across the microsieve filter is much lower compared to other size based filtering systems. A low pressure results in less force and less damage on the captured cells." [55]

Despite the construction of the VYCAP filter, the microscopy of the microsieves revealed damage to the cells. VYCAP normally uses blood fixed in Transfix tubes, which can withstand a higher pressure [64]. With fresh EDTA blood, the pressure needs to be lowered to 5-25 mbar. This makes the filtration go much slower, with a risk of blood clogging during the procedure. If the pressure is higher than 25 mbar for fresh blood, there is a risk of damaging the cells. The first validation experiments showed major damages to the cells, as if they were almost smudged out over the filter. The cells had malformed nuclei in addition to weak membrane staining of leukocytes (CD45). This type of damage occurs when non-fixed cells experience too much pressure under the presence of a high flow rate [65]. It was therefore attempted to lower the pressure and stop the pump before it started filtrating air. This resulted in less damage, but nonetheless damaged cells. The conclusion is that size-based filtration with VYCAP does not work well with EDTA blood. It was also concluded that fresh ZR75-1 cells do not tolerate filtration, likely due to deformability of the cell line. This in turn could indicate that real CTCs are more vulnerable to filtration, but there is not necessarily a correlation. Due to observed damaged to the cells, the decision was made to switch from fresh EDTA blood to CellSave blood. When using CellSave tubes, the cells are fixed in the blood sample tube and thus tolerate the filtration better. There was still some damage observed after using CellSave tubes, though considerably less than with EDTA tubes.

Samples can be left in CellSave blood collection tubes up to 96 hours before filtration [66], but no possible lower limit is given as an indication of how long a sample should be fixed before starting filtration. It would be interesting to discover the optimal time for a sample to stand in a CellSave tube before filtration. The results pointed to a positive outcome for a sample to stay overnight. Correspondence with a VYCAP employee indicated a recommended fixation time for CellSave tubes of 24 hours in room temperature [67]. The staining and filtration protocol adapted to the CancerID program, optimised for CellSave fixed whole blood, advices that CellSave blood should be used within 48 hours after the draw to avoid clogging of the filter [22]. The appearance of large clusters, resulting from long fixation-time of the sample, will fill the pores of the filter more rapidly and the CTC recovery will be lowered. Longer fixation increases the risk of clogging of the microsieve, as smaller cells can turn too stiff to pass the pores of the filter. VYCAP recommends the use of the fixative called Transfix, which is milder than the fixative in CellSave blood collection tubes [67].

An aspect of pressure specifically connected to the VYCAP equipment was that the pressure varied greatly for some samples during filtration, without adjustment. The reason for this is unknown and it is not clear whether this affects the outcome of the enrichment or contributes to possible damage of cells. The flow rates were observed to fluctuate between samples. According to the staining and filtration protocol adapted to Cancer ID program, the flowrate is dependent on the sample age and the donor [22, 68]. Another problem with the equipment was the length of the microsieves. In order

to microscope properly, an adapter to the microsieves was needed. It is, however, possible to obtain longer microsieves that should be suited for microscopy without the need of an adapter. The construction of the microsieve makes it hard to microscope and take pictures with the 40x and 100x objectives.

4.3 Staining and detection – separating CTCs from other cells

A consequence of size-based filtration that reflects on the staining and detection of cells is that size-based filtration results in the enrichment of other large cells present in blood, such as different types of immune cells [47, 69]. This leads to low purity of the sample. One encountered problem during the project was that many of these large immune cells that get caught in the microsieve filter are weakly positive for keratin, in addition to being CD45+. This makes it more effortful to distinguish between the immune cells and the captured CTCs. The category "potential CTCs" in the patient samples probably contain many such cells (see Table 12, Section 3.3). Some of the potential CTCs could be eosinophilic granulocytes since these have a double nucleus. Many immune cells are polymorphonuclear. For instance, the nuclei of neutrophils consists of three or more separate lobes [70]. Many of these cells were KRT+ but they were easily rejected as non-CTCs because of their characteristic nuclei. The cytokeratin AE1/AE3 antibody binds to the keratins 1-8, 10, 14-16 and 19 [71, 72], while the cytokeratin C11 antibody binds to the keratins 4-6, 8, 10, 13, and 18 [73]. According to proteinatlas.org [74], KRT5 is used as a prognostic marker for breast cancer, and it can also be expressed on plasmacytoid dendritic cells. However, most leukocytes do not express keratin [74]. The weakly binding of the keratin antibody to leukocytes could be a general unspecific phenomenon. It is possible to use isotype control antibodies for analysis of the immunological staining [75]. Further experiments could apply this to determine if the staining is unspecific or if these leukocytes actually contain keratin. Some staining of the microsieve filter was observed which disturbed the counting of CTCs in patient samples. The background for this filter-staining is unknown.

In a study by S. A. Joosse et al. [24], they show that the keratin markers that are currently being used for detection of CTCs can be downregulated when metastasis proceeds [24]. The antibodies we use in the current study may not detect CTCs with altered keratin expression. These CTCs are however associated with progression of metastasis in breast cancer patients [24], and thus of interest to detect. This points to possible benefits of using a mix of different antibodies that are relevant for CTC detection. When filtrating solely ZR75-1 cells, there was no cross-signal from the keratin staining in the other channels. However, observations from patient samples indicates some unspecific binding of the CD45 antibody and the CD61 antibody. Although slight unspecific binding of the CD61 antibody was seen in the optimalisation experiments, the unspecific binding of CD45 had not been observed earlier. The unspecific binding of the CD61 antibody was primarily seen when using the 100x objective.

During the project we encountered problems with the isolation and staining of platelets. The main problem was that we could not find the platelets at first. The protocol used for isolation, fixation and staining of platelets was modified several times and it required the removal of one washing step to guarantee platelet recovery. After modifying the protocol to be able to successfully isolate the platelets, it was possible to look at the staining. In the end, we found out that the intensity of the staining of platelets for the different concentrations of CD61 antibody was quite equal. Therefore, the decision was made to use a 1:50 concentration, which is the amount recommended by the manufacturer [76]. It was also observed some unspecific binding of the CD61 antibody to keratin positive cells. The use of a lower concentration of CD61 antibody could help to reduce the unspecific binding in future experiments.

4.4 CTC DETECTION IN PATIENT SAMPLES

The larger the number of analysed cells with an immunofluorescent staining, the higher the influence of non-specific binding. For rare cell detection, a non-specific background of 0,01% would result in detection of 100 false CTCs after analysis of 1,000,000 leukocytes [58]. There is a fine balance for determining a threshold for detection of CTCs, while trying to avoid false-positives [6].

According to most studies, the CTC count in patients is generally quite low even in patients with metastatic disease [19]. However, some cases with very hight CTC count have been observed – as much as up to 100 000 CTCs in a nonmetastatic primary breast cancer patient [77, 78]. Almost all patient samples attained in this project had detectable CTCs (9 out of 10 patient samples). The patient samples show a diversion in the number of detectable CTCs, varying from 0 up to 35. However, the number of potential CTCs was considerably higher (179 at its highest). It seems like the number of potential CTCs using size based filtration was significantly higher than when using the MINDEC method [63]. It is likely that the number of false positives is higher with size-based filtration technologies than with other enrichment methods, due to the lack of specificity and the low purity [79]. This is supported by the fact that recovery rates for size-based experiments have been lower compared to other methods, while detection in patient samples have been higher. Comparing recovery rates from spiking experiments performed with different enrichment methods, many methods show higher recovery rates than size-based filtration [47]. However, the number of CTCs found in patients often seem to be higher when using size-based filtration than when using for example EpCAM based approaches. In an article by Huebner et al. [80], they compared the number of CTCs enriched from mBC patients using CellSearch® technology and a filtration-based method. They found the CellSearch® system to have a CTC positivity rate in 56,7% of patients while the filtration-based method had a CTC positivity rate in 66,7% of patients [80].

The first patient samples obtained were collected in EDTA tubes, the rest in CellSave tubes. This may have impacted the results, as the validation experiments suggests that fixed blood leads to a higher recovery. The general impression from the results is that a lower number of CTCs were found in patient samples obtained in EDTA tubes. This is however not certain since the highest number of CTCs detected (35 CTCs) also occurred in an EDTA tube and the lowest number of CTCs (0 CTCs) occurred in a patient sample taken with a CellSave blood collection tube.

4.5 INTERACTION BETWEEN CTCS AND PLATELETS

It was possible to find platelet attachment to single CTCs in six of the nine (67%) patients where CTCs were detected. The percentage of CTCs with platelets varied greatly between these patient samples, ranging from 5,7% to 70% of CTCs having platelets. In addition to the CTCs with confirmed platelets, there were a few platelets that lay close to other CTCs. A threshold distance was set to 0,3 µm to determine which platelets could be classified as interacting with CTCs or not. The average percentage of CTCs with platelets out of the total number of CTCs for all patient samples was 25,5%. This number is comparable to the numbers in a study by Lauren Brady et al. [81] that also utilised size-based filtration for enrichment of CTCs. They found platelet cloaking of circulating tumour cells in 29,5% of patients with metastatic prostate cancer. The CTCs were isolated with the ScreenCell® platform with a filter consisting of a combination 6,5 and 7,5 µm pores that are distributed randomly [81, 47]. Additionally, CTC enumeration, detection and classification performed with a computational cell imaging system by Chai et al. [82] revealed that 30,4% of patients with metastatic castrate-resistant prostate cancer had platelet coated CTCs [82]. In one study the number of platelet markers found on CTCs in patient samples was found to be as high as 43,8%. This study also found that as much as 90,7% of CTCs in patients with unresectable pancreatic cancer were coated with platelets and that these

patients also had a very poor prognosis. They utilised a centrifugal microfluidic device for isolation of CTCs [83]. In comparison, the results from Julie Kloster Snekkevik [63], who isolated CTCs with the MINDEC negative depletion method, have found the interaction of platelets with CTCs in patients with metastatic breast cancer to be significantly lower [63].

The presence of platelets on the surface of CTCs has recently been studied as a predictive biomarker for patients with metastatic cancer disease [82]. Platelet interaction with circulating tumour cells is thought to have a prognostic value, and interaction with platelets have been shown to correlate with a poor prognosis and rapid metastasis in unresectable pancreatic cancer and in metastatic castrate-resistant prostate cancer [83, 82]. However, the interaction between CTCs and platelets is not thoroughly mapped nor understood yet. The potential therapeutic effect of targeting platelets functions is being investigated. By blocking tumour-specific platelet functions, Xu et al. [39] discovered that EMT was supressed, and metastasis reduced [39]. Although these findings show promise, much remains to be determined. In another trial [84], the effect of inhibiting platelet function in patients with mBC was explored. The results showed that the treatment did not have any effect on the CTC count [84]. The interaction of platelets and CTCs seem to be complex and involves many signalling pathways. These interactions could be a target for possible treatment strategies of cancer [21].

All the clusters found in the patient samples in this project had platelets in them. This is similar to what has been observed in other studies. Lim et al. found associated platelets in 80% of CTC clusters [83]. They also found CTC clusters to correlate with a worse prognosis than single CTCs [83]. CTC-clusters have a higher metastatic capability than single CTCs [84]. If platelets are more prone to attachment in CTC clusters, and the clusters in turn correlate with poor prognosis – this could indicate that CTCs with platelets have a larger chance of survival in the circulatory system. If the clusters survive longer, due to protection of platelets, they can form metastases which will in turn lead to poor prognosis. Wang et al. [85] have investigated the importance of CTCs and CTC-clusters in patients with metastatic breast cancer. They enriched CTC-S and CTC-C using the CellSearch® system (positive enrichment) and found that large-sized clusters correlated with a higher mortality in these patients [85].

The observations in this study were in that the CTCs had one or two platelets attached to them, which would not protect the CTCs in the blood. This could indicate that other platelets are removed during the enrichment procedure which in turn suggests that the interaction between platelets and CTCs is quite weak. It is however not possible to draw any conclusions based on the low amount of data in this project. How suitable size-based filtration is for detection of platelets on CTCs is also dependent on the strength of the possible interaction between a CTC and platelets. If the platelets bind very strongly to the CTC, size-based filtration would probably be a very good way to detect CTCs with platelets on. If, on the other hand, the interaction is weak, the platelets might get “flushed away” during filtration and go through the filter, leaving the CTC behind.

Since the presence of platelets on single CTCs occurred in about half the number of patients, it is hard to draw any conclusions as to the frequency of platelet-CTC interaction. It is possible that this can be something patient-independent. More patient samples are required to be analysed. It is however possible to conclude that size-based filtration can be used for detecting platelets on the surface of CTCs. The results from the detection of platelet-covered CTCs using VYCAP’s size-based filtration are comparable to results of other studies using size-based filtration, in addition to techniques based on computational analyses. The percentage is slightly lower than in the detection of platelet-coated CTCs utilising a centrifugal microdevice [83]. On the other hand, it seems to be much higher than the number retrieved from results of negative depletion [63]. With this in mind, it is possible that physical property-based enrichment of CTCs is more advantageous for detection of platelet-coated CTCs than biological

property-based enrichment methods. The conclusion is that CTC platelet interaction occur, but the number of samples in this study is too low to give a reliable estimate of how often it occurs.

4.6 Further research

Much remains to be determined regarding size-based filtration as a method for enrichment and detection of CTCs. The next step in optimising the protocol for filtration would be to validate its performance at different conditions, such as pressure and cell line size. Experiments should address the pressure and time of filtration. Filtration should be validated at different millibars to find the optimal pressure for both EDTA blood and CellSave blood respectively. In addition to this, the VYCAP method should be validated based on the conditions given in the VYCAP protocol, such as the use of TransFix blood collection tubes and the staining solution of VYCAP. The use of CD16 antibody is recommended in addition to CD45 antibody to brighten the staining of immune cells [64].

More patient samples need to be analysed to better see the connection, if there is any, between platelets and circulating tumour cells. It is hard to determine how many of the CTCs with platelets in this project were actually interacting and how many were coincidental. The number of false positive CTCs in size-based filtration should be further investigated. One idea could be to use single-cell analysis to analyse the individual isolated cells and find out if they are indeed CTCs or other cells. The work done by Lapin at the laboratory of molecular biology at SUS, using the size-based filtration to extract mRNA, could give further insights into the possibilities and limitations of this technique. The mRNA analysis could confirm if the detected cells are CTCs or if they are mostly immune cells. Further research should also determine if size-based filtration is more suitable for detection of CTC clusters, and the interaction of platelets with such clusters.

Future experiments should focus on determining the nature of CTC-platelet interaction. More details about the interaction of platelets with CTCs need to be unravelled. It should be investigated how platelets bind to and interact with CTCs in the first place. It should also be determined how strong or weak the interaction is. If the interaction is weaker, it could be favourable to use a stronger fixative before isolation to preserve the interaction and avoid the platelets detaching from the CTCs during enrichment. It would also be interesting to understand how platelets aid the circulating tumour cells. Can platelets mediate the growth and survival of CTCs by excretion of platelet derived growth factors? Understanding of these interactions is vital for finding therapies to target them.

In the future it would be interesting to apply other CTC enrichment methods to the same patient group to determine if one can find platelets on CTCs using those methods and decide which method is the better approach for finding such interactions. The frequency of platelet-CTC interaction should be determined. It is also thinkable to investigate the interaction of platelets and CTCs in the metastatic process by following patients over time. It could be useful to obtain information to see how the treatment effects the CTC count and the ratio of platelet-covered CTCs to CTCs without platelets. It is also interesting to investigate the prognostic value of CTC-clusters.

Potentially, since size-based filtration is independent of antigen expression – the technique could be used to investigate the ratio of epithelial to mesenchymal CTCs. If the primary tumour of the patient is determined to be of epithelial origin and these cells have an epithelial phenotype, it would be of interest to find out how many of the CTCs go through EMT and how this number relates to the disease progression and metastasis.

5 CONCLUSION

This project has established that it is possible to enrich CTCs from patients with metastatic breast cancer using size-based filtration. A subset of the CTCs enriched from patient samples had detectable platelets on them. This was a relatively low number, but the conclusion is that platelet-CTC interactions do occur. We also found some CTC-clusters in the patient samples, and these were more frequently associated with platelets. Further research and more patient samples are necessary to establish how frequent the interactions between platelets and CTCs are.

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7 APPENDIX

Appendix A – T-staging

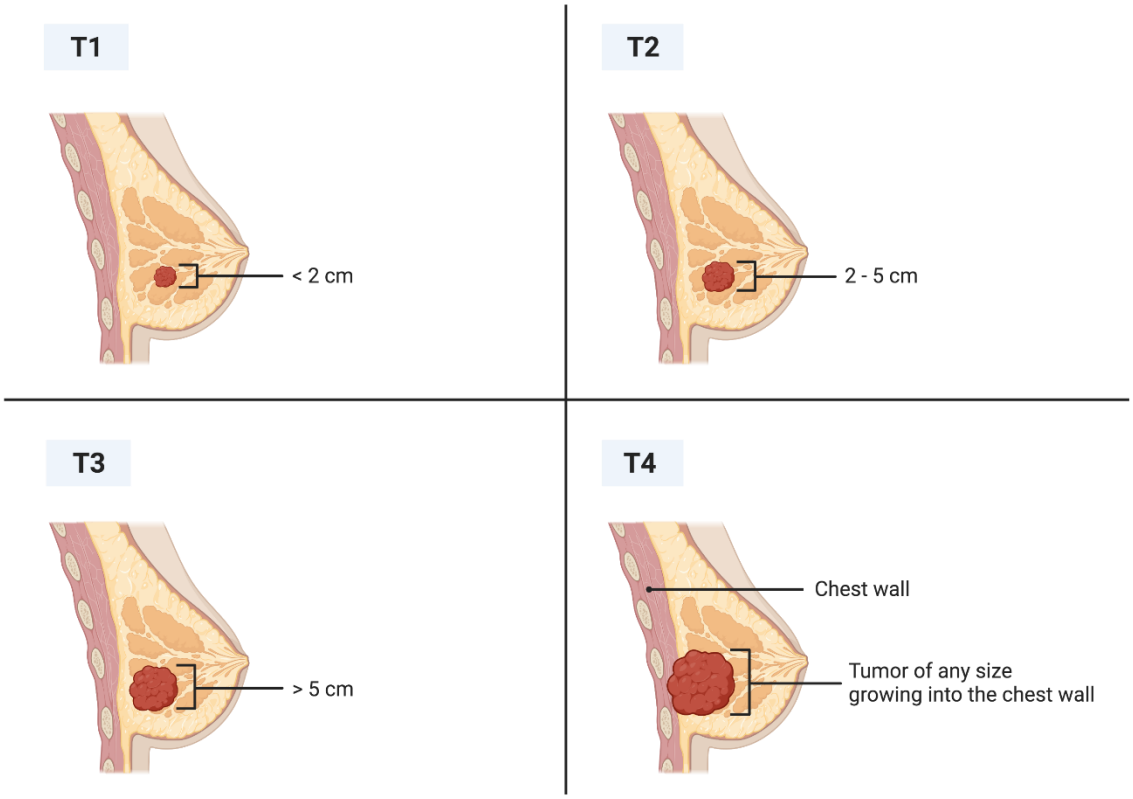

Figure 19. T-staging. The figure is showing the different T stages T1-T4. T1 describes a small (<2 cm) localised non-invasive tumour, T2 and T3 describe larger tumours and T4 describes an invasive tumour that is growing into the chest wall. The figure is adapted from "Breast Tumor Size Chart", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

Appendix B – Overview of the different cell types in human blood

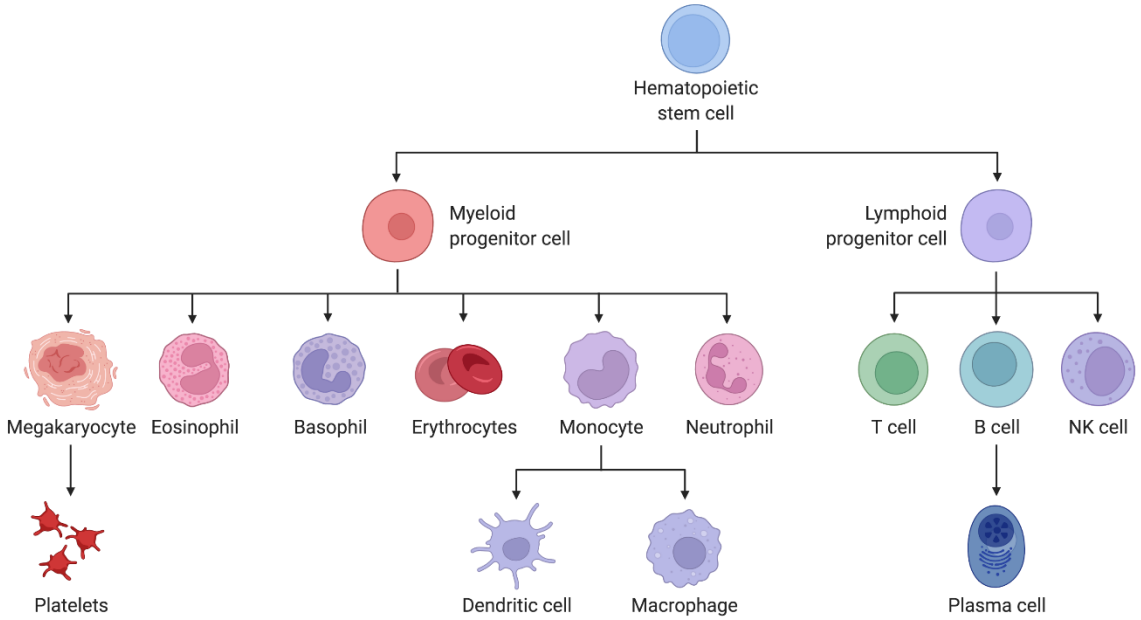

Figure 20. Stem cell differentiation. This figure shows an overview of the different blood cells. The figure is adapted from "Stem Cell Differentiation from Bone Marrow", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

Appendix C1 - Protocols

PROTOCOL IX: FILTRATION OF ZR75-1 CELLS

In this procedure, ZR75-1 cells are filtrated with VYCAP size-based filtration technology.

1. Split the ZR75-1 cell line and count cell density.
2. Add a volume corresponding to 10 000 ZR75-1 cells to a tube with 10 ml of VYCAP buffer (PBS/1% BSA). Mix well.
3. Set the pressure on the filtration pump to 15 mbar.
4. Insert a filtration unit into the pump and check that the o-ring is intact, and that the unit is fully inserted.
5. Transfer the ZR75-1 cells/PBS solution to the filter unit.
6. Turn on the pump and be prepared to stop the pump just before all the fluid is gone. The filtration will be quick.
7. Wash the filter with 1 ml PBS/1% BSA. Stop the pump immediately before all the fluid is gone. Repeat this washing step.
8. Remove the filter unit from the pump and then remove the microsieve part from the filter unit.
9. Attach an absorber to a staining holder and place the microsive in the staining holder.
10. Press the upper part of the microsieve down towards the absorber to remove the remaining fluid on the filter. If liquid remains on the edge, it can be removed carefully with a paper towel. Avoid drying of the filter by proceeding immediately to the next step in the protocol.
11. Wash the filter with 75 µl PBS/1% BSA. Remove the washing solution by pressing down towards the absorber. Repeat the washing.
12. Apply 100 µl of 4% paraformaldehyde in PBS to the filter. Incubate for 20 minutes at room temperature. During this time, make a mastermix consisting of 53 µl in total. Mix well.

    - 40 µl PBS with 0.1% saponin
    - 10 µl FcR blockage solution
    - 0.5 µl 0.1 mg/ml DAPI
    - 0.5 µl Anti-Human Pan Cytokeratin AE1/AE3 eFluor 570
    - 0.5 ul Anti-Human Pan Cytokeratin CK-11 PE
    - 1 µl Anti-CD45 APC
    - 1 µl CD61

13. Remove the paraformaldehyde by pressing down.
14. Wash the filter with 75 µl PBS/1% BSA.
15. Add 53 µl of antibody solution with blockage and saponin to the filter. Make sure that the entire filter is covered with liquid. Incubate for 20 minutes at room temperature without light.
16. Remove the staining solution by pressing down onto the absorber.
17. Wash the microsieve with 75 µl PBS/1% BSA and removed the washing solution by pressing down to the absorber.
18. Repeat the washing, but now incubate for 5 minutes at room temperature without light before removing the washing solution. Press extra long against the absorber to remove all fluid.
19. Allow the filter to dry for 10 minutes at room temperature without light in the holder.
20. Turn the filter upside down in the staining holder. Add 15 µl of mounting medium on the back of the filter. Carefully place a VYCAP 8 mm cover glass. Do not press.

21. Turn the filter holder over and carefully apply 13 µl of mounting medium on the top of the filter, approximately in the middle. Gently place an 8 mm cover glass onto the filter. Do not press.
22. Incubate for 10 minutes at room temperature without light. Store in a refrigerator (dark).

PROTOCOL X: SCANNING IN METAFER 4

The following was done for scanning of validation slides and in Metafere 4.

1. Use the program Metafer 4, RCDetect.
2. The settings used in Classifier Setup can be found in Appendix C3. (Classifier = CTC2022) The focus Parameters used were FL20 and 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

3. Place the slides in the slide holder under the microscope. Click on Setup and enter the name of the slide. Use Mode = RCD, Classifier = CTC2022, Search Window = Manual, Max. Cnt = 10 000
4. Use the Search function to determine the area of the slide and to scan the sample.
5. For counting of CTC, use Training: Classify fields.

Appendix C2 – Metafer 4, Classifier Setup

```
Metafer4   V3.5.12

RCD Classifier, Version 3.5.12   5/25/22 18:43:35
--------------------------------------------------------------------------------
Classifier Name :
   Cell
Classifier Description :
   RCDetect CTC classifier, 10x, red label., CD45
Focus Parameters :
   FL10
--------------------------------------------------------------------------------
Color Channel Number :
       3       2       1
Exposure Mode :
       1       1       1
Maximum Integration Time (in sec) :
    0.416676    0.625024    0.625024
Transmitted Light Color Capturing Mode (0..1) :                        [3.3.110]
       0
Use Automatic Objective Change ? :                                     [3.5.101]
       0
Search End Objective Number :                                          [3.5.106]
       0
Use Auto-Oiler ?                                                       [3.5.102]
       0
Enable Classifier Linking ? / Link Loop Mode ? :                  [3.5.101/104]
       0       0
Link to Mode :                                                         [3.5.101]
       2
Link to Classifier :                                                   [3.5.101]

Is Fixed Search Window Enabled ? :                                     [3.5.102]
       0
Fixed Search Window Type :                                             [3.5.102]
       0
Fixed Search Window Size / Selection :                                 [3.5.102]
       0
--------------------------------------------------------------------------------
Minimum Signal Contrast :
       0
Lower Limit of Signal Intensity :
       0
Required Maximum Object Area :
      80
Number of Objects to exclude :
      60
Maximum Area Sum Remaining Objects :
     780
Counterstain Object Threshold :
      30
Minimum Counterstain Area :
       1
Maximum Counterstain Area :
      90
--------------------------------------------------------------------------------
Extend Counterstain Mask by :
       3
Relative Center Radius (%) :
      10
Maximum Center Intensity (%) :
       0
Concavity Counterstain Threshold :
      30
Maximum Concavity Area (%) :
       0
--------------------------------------------------------------------------------
Microscope Magnification :
   10.00
```

```
Overlap of Image Fields (X,Y) :
      20      20
Minimum Distance Positive Objects :
      10
Gallery Image Size :
       1
Region of Interest Size :
     560
CCD Camera Gain Factor (%) :
     100
Last TL Light Level :
       0
--------------------------------------------------------------------------------
Delay Long XY Movements :
     500
Delay Short X Movements :
     180
Delay Short Y Movements :
     180
Delay Cube/Filter Change :
     100
Is Fast Filter Change Available ? :
       0
Microscope Configuration Number :
       1
--------------------------------------------------------------------------------
Object Threshold Cell Counting :
      20
Minimum Object Area Cell Counting :
      40
Maximum Object Area Cell Counting :
     500
Maximum Concavity Depth :
      40
--------------------------------------------------------------------------------
Use 2. Signal Condition :
       1
2. Signal of Cell :
       1
2. Signal Greater Than :
       1
2. Signal Threshold :
      20
--------------------------------------------------------------------------------
```

Appendix D1 – Results, Immunofluorescent staining of cells from optimalisation of protocols

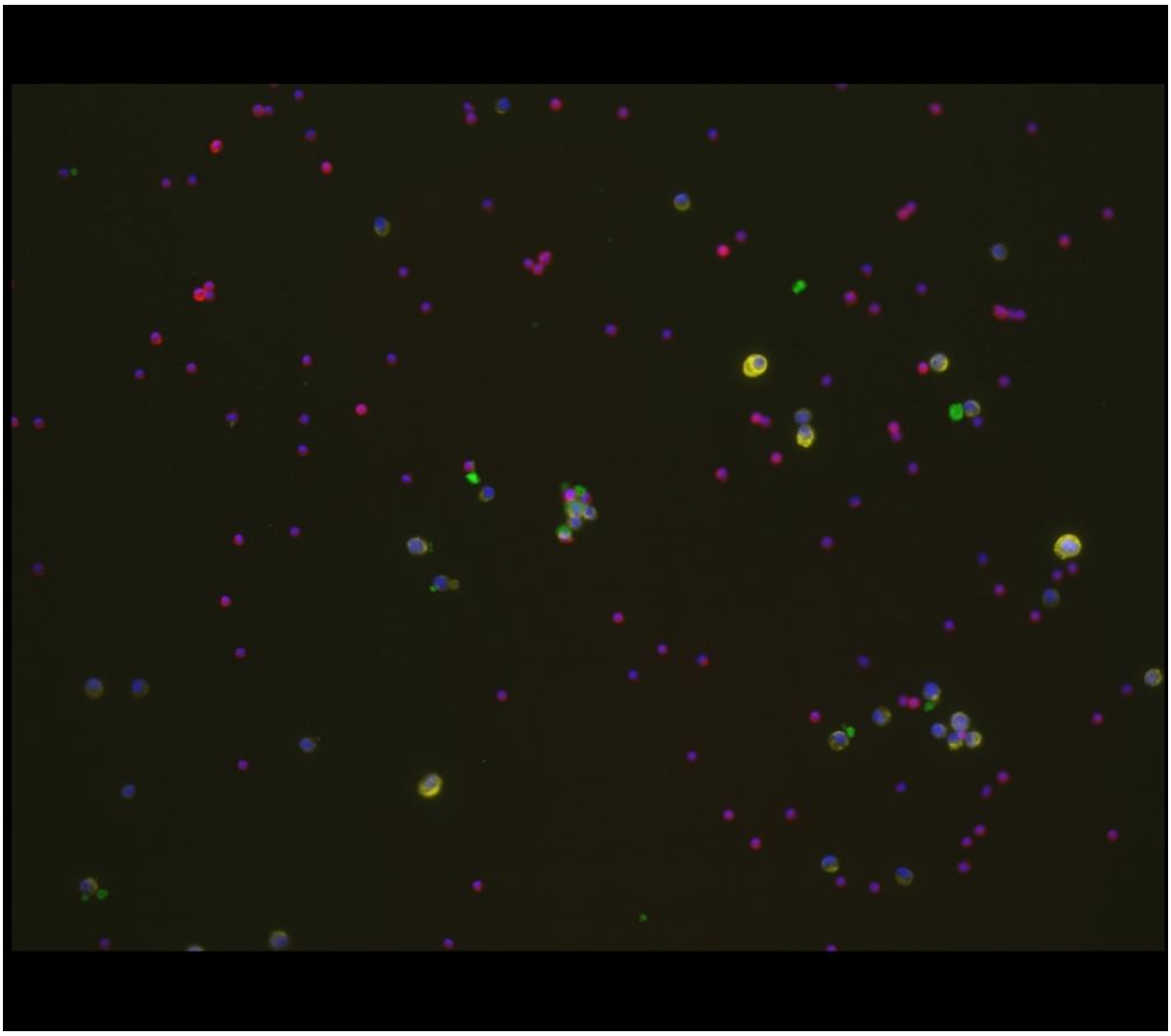

Figure 21. Immunofluorescent staining of cancer cells, leukocytes, and platelets. The cells are stained with antibodies against CD45 (red), keratins (yellow), and CD61 (green). Cell nuclei are stained with DAPI (blue). This picture was taken in combination with the experiment for determination of the optimal immunofluorescent staining of cancer cells with antibodies. The picture is from tube number 8 that contains a combination of the two keratin antibodies. Platelets were observed to aggregate. The picture was taken in the ISIS software using the 10x objective.

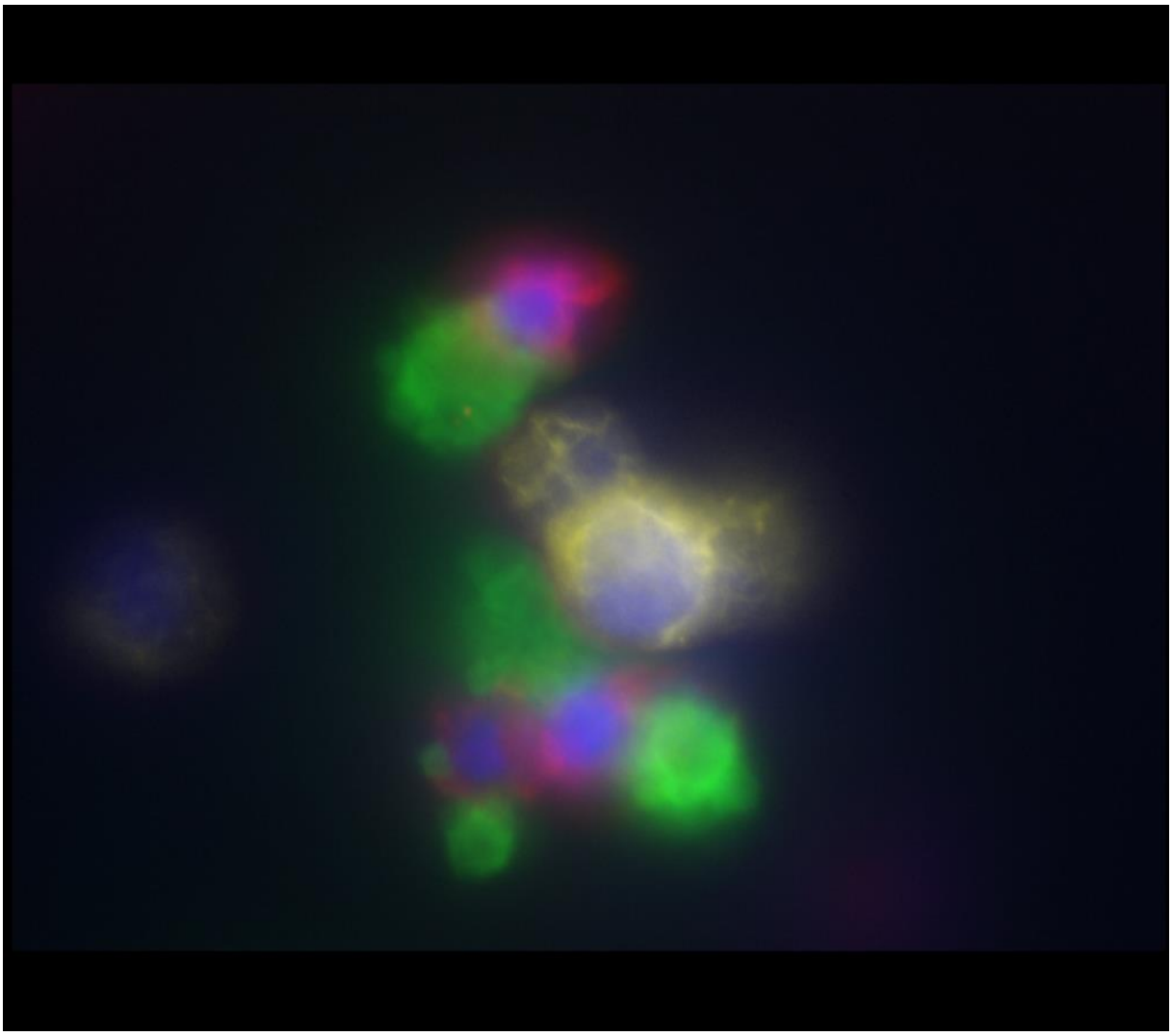

Figure 22. Immunofluorescent staining of leukocytes, ZR75-1 cells and platelets. The cells are stained with antibodies against CD45 (red), keratins (yellow) and CD61 (green). The nuclei of the cells were stained blue with DAPI. This picture is from the experiment with staining of cancer cells using two antibodies. Some aggregation of platelets has occurred. The picture was taken in the ISIS software using the 100x objective.

Appendix D2 - Results, Validation experiments, damaged cells

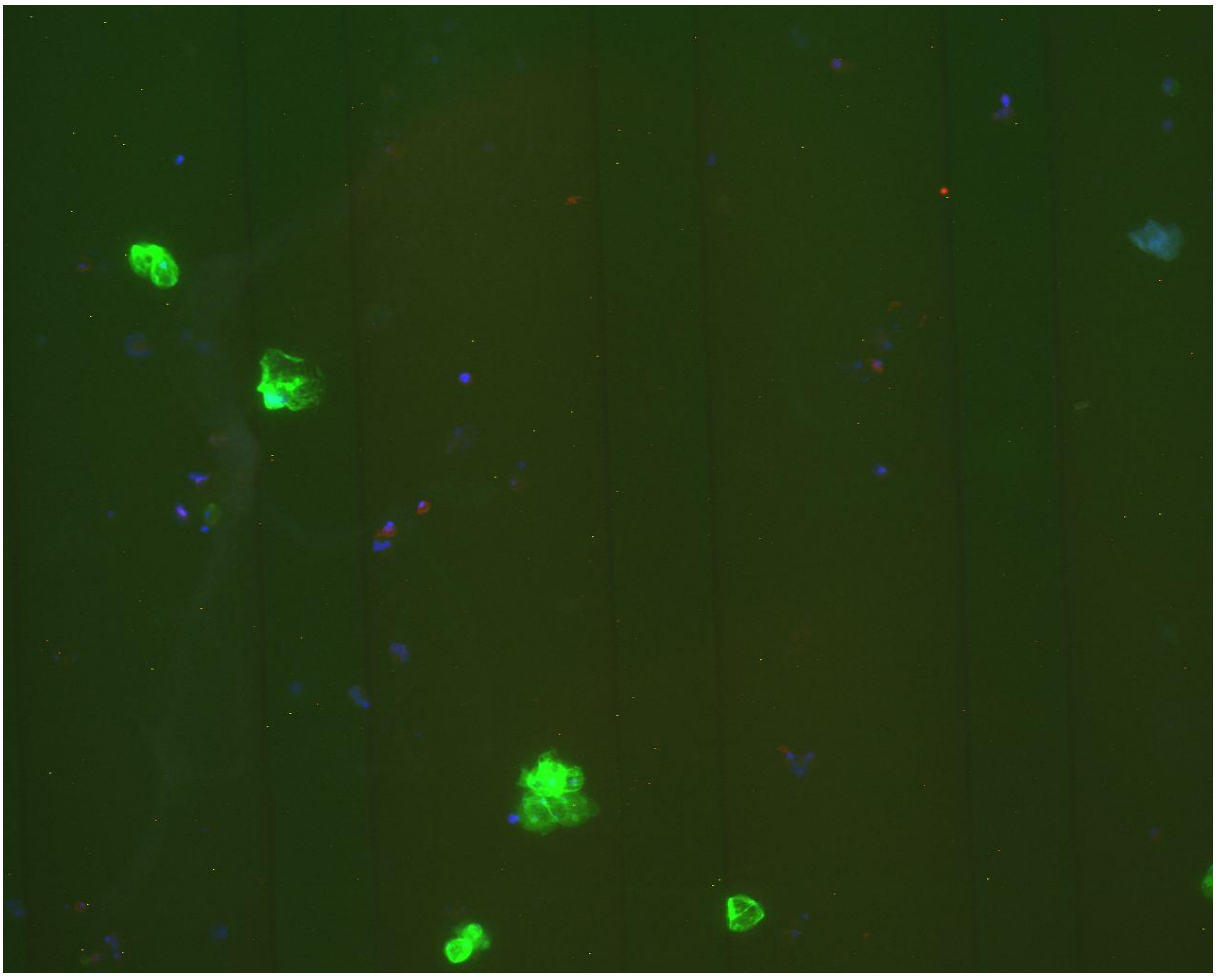

Figure 23. Cells damaged by filtration. Immunofluorescent staining of leukocytes and ZR75-1 cells using antibodies targeted against CD45 (red), and keratin (green). The cell nuclei are stained with DAPI (blue). This picture shows cells from one of the validation experiments utilising the VYCAP size-based technology for enrichment of spiked-in ZR75-1 cells. This validation was performed using fresh EDTA blood and there were extensive damages to the cells due to filtration. The picture is taken in Metafer4 RCDetect scanning program.

Appendix D3 – Results, Filtration with CellSave Blood

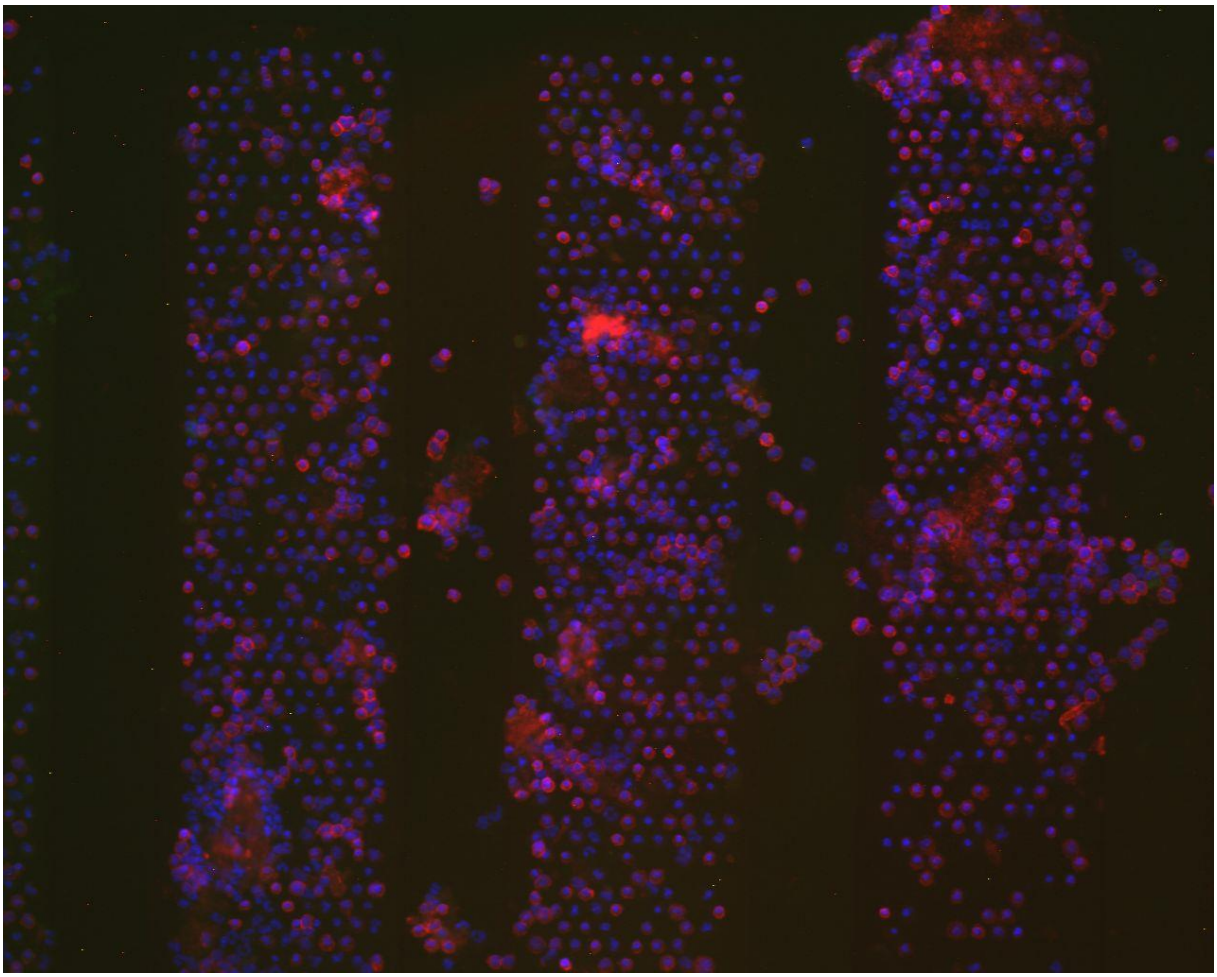

Figure 24. Filtration of CellSave blood. This picture shows cells filtrated after 2 days of being in a CellSave tube. Immunofluorescent staining of leukocytes, CTCs and platelets with antibodies targeted against CD45 (red), keratins (green) and platelets (not shown). The nuclei of the cells were stained with DAPI (blue). The picture was taken in Metafer4 RCDetect scanning program using the 10x objective.

Appendix D4 – Results, Calculations

Percentage of platelet-associated CTCs in patient samples.

A_1: $\frac{2}{35} \times 100\% \approx 5{,}7\%$

A_2: $\frac{6}{24} \times 100\% \approx 25{,}0\%$

C_2: $\frac{9}{23} \times 100\% \approx 39{,}1\%$

E: $\frac{7}{10} \times 100\% = 70\%$

F: $\frac{11}{27} \times 100\% \approx 40{,}7\%$

G: $\frac{1}{8} \times 100\% \approx 12{,}5\%$

Average percentage of platelets-associated CTCs in these samples:

$\frac{5{,}7+25{,}0+39{,}1+70{,}0+40{,}7+12{,}5}{6} \approx 32{,}2\%$

Appendix D5 – Results, Staining of the microsieve filter

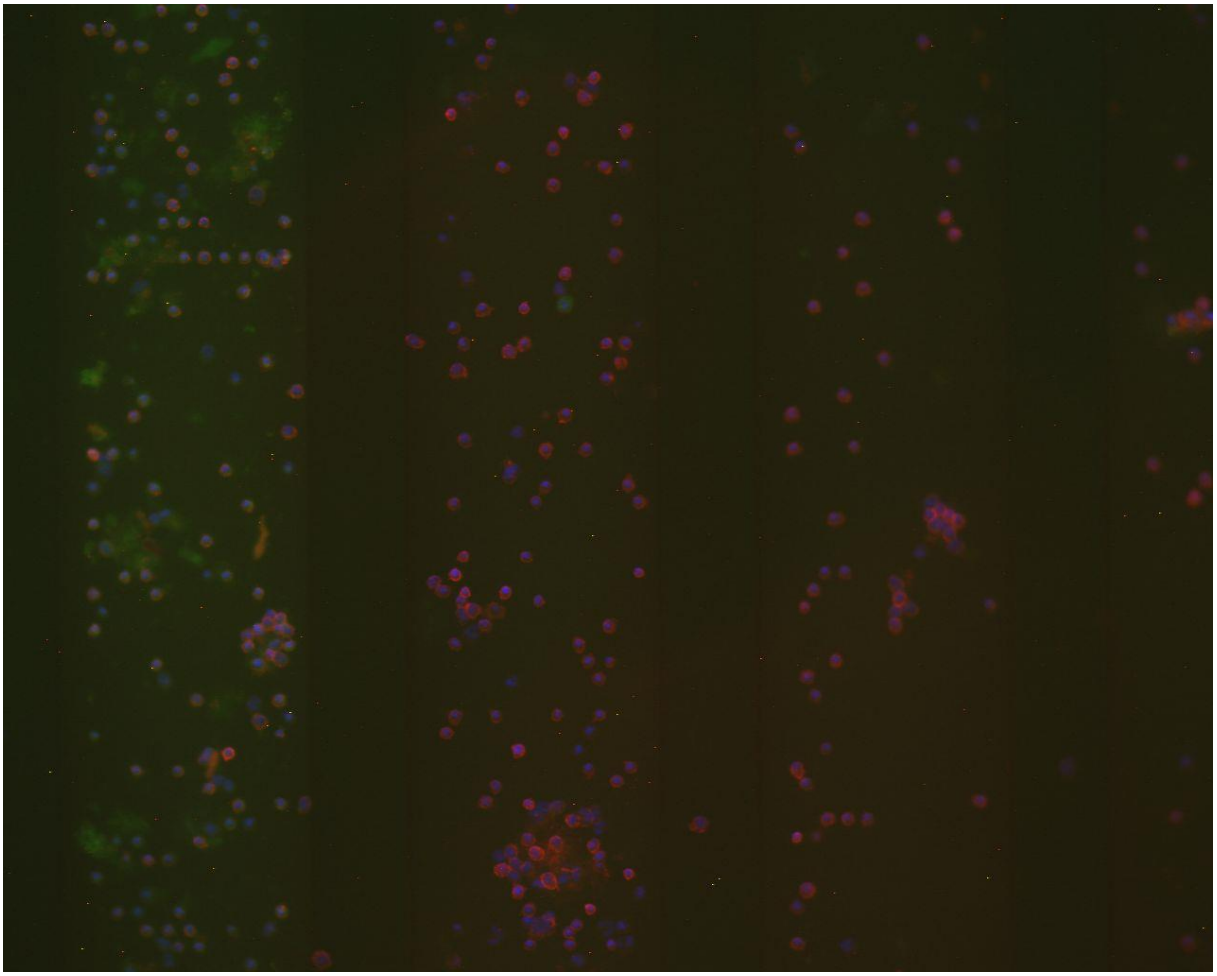

Figure 25. Staining of the microsieve filter. Immunofluorescent staining of leukocytes, CTCs and platelets with antibodies targeted against CD45 (red), keratins (green) and platelets (not shown). This picture shows staining of the microsieve filter. The green colour makes it harder to detect CTCs. This picture is taken in Metafer4 RCDetect scanning program.

Appendix D6 – Results, Overview of filtration time and pressure of different patient samples

Table 13. Filtration of patient samples. Some samples were filtrated on the same pressure the whole time. For other samples, the pressure started low and was raised during the filtration. This is often the case for the samples taken in EDTA tubes. For one of the samples in CellSave tubes, the pressure was set high at first and then lowered because the filtration was to fast at that pressure. According to VYCAP protocol the pressure should be so that it takes between 1 and 3 minutes to filtrate 1 ml of blood.

Patientmple	Pressure (mbar)	Time (min)	Total filtration time (min)
A_1	10 15 20	15 15 25	55
A_2	75	5,5	5,5
B_1	10 15 20	15 5 25	45
B_2	75	6	6
C_1	20 25	30 5	35
C_2	75	4	4
D	100 50	1 4	5
E	50	7	7
F	50 75	6 2	8
G	75	4	4

Appendix D7 – Results, Additional pictures of CTCs with platelets from patient samples

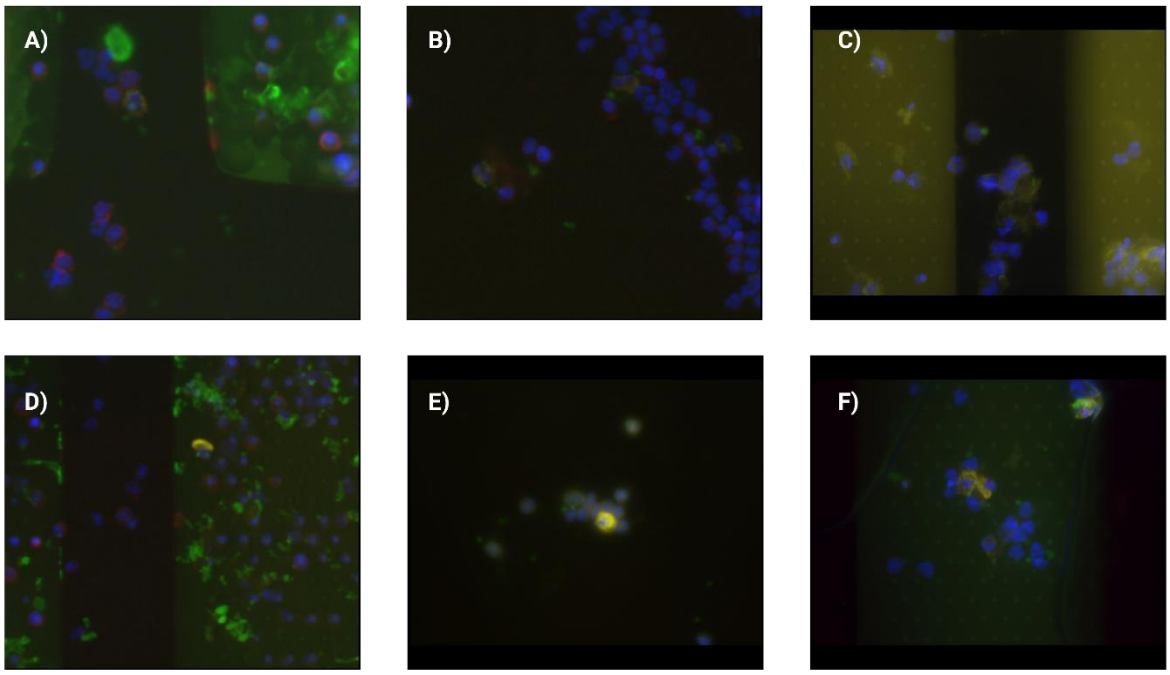

Figure 26. Platelet Interaction with Circulating Tumour Cells. Immunofluorescent staining of leukocytes, CTCs, and platelets with antibodies against CD45 (red), keratins (yellow), and CD61 (green). The nuclei of the cells are stained with DAPI (blue). A) Platelet-CTC interaction. The picture was taken in the ISIS software using the 10x objective and the zoom function. B) Platelets interaction with possible CTCs. The picture was taken in the ISIS software using the 40x objective. C) Platelet Interaction with Circulating Tumour Cell. Extensive staining of the filter can be seen. The picture was taken in the ISIS software using the 40x objective. D) Two platelets attached to a circulating tumour cell. The membrane of the cell appears to be damaged. The picture was taken in the ISIS software using the 10x objective and the zoom function. E) Platelet interaction with CTCs. The picture was taken in the ISIS software using the 40x objective. F) Platelet interaction with a circulating tumour cell. The picture was taken in the ISIS software using the 40x objective. The figure is created with BioRender.com.

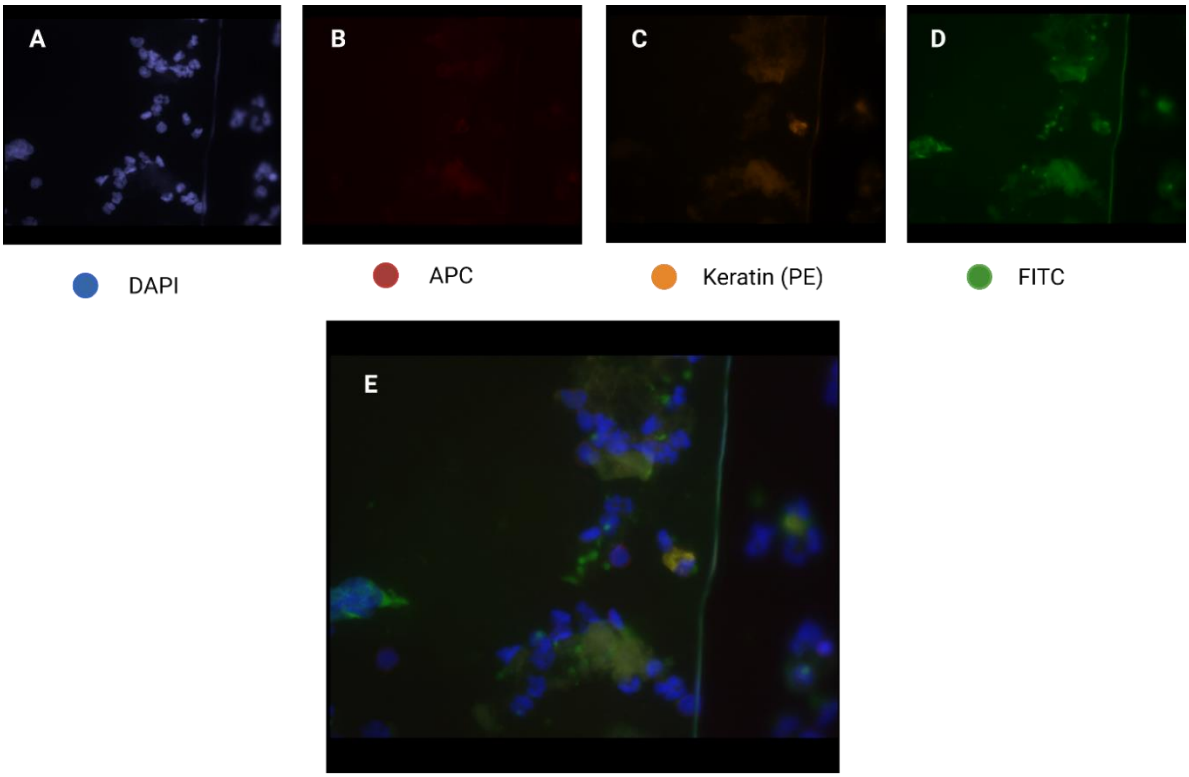

Figure 27. Platelet interaction with a circulating tumour cell. A) Staining of the nuclei with DAPI (blue). B) Staining of leukocyte membrane with antibody against CD45 (red) is seen in the APC channel. C) Staining of CTCs with keratin antibodies is seen in the PE channel. D) Staining of platelets with CD61 antibody is seen in the FITC channel. E) Merged colours. It is possible to notice the unspecific binding of the CD61 antibody. The figure is created with BioRender.com.