UNIVERSITY OF TWENTE.

Master thesis
February - October 2017

Identification of circulating tumour cell subpopulations

Author:
Froukje Deelstra
Biomedical Engineering

Examination committee:
Prof. Dr. L.W.M.M. Terstappen
Dr. J. Prakash
Dr. J.F. Swennenhuis
S. de Wit

MIRA
BIOMEDICAL TECHNOLOGY
AND TECHNICAL MEDICINE

MCBP

Preface

I would like to thank Prof. Dr. L.W.M.M. Terstappen for giving me the opportunity to fulfil my master thesis in his research group Medical Cell BioPhysics. I would also like to thank my daily supervisors Joost and Sanne. Their insights, suggestions, ideas, help and critical view have made this period very interesting. Furthermore, I would like to thank everybody for their interest and support.

This paper is the result of a nine month long graduation process. It cannot express the long days spent in the lab, the hope for good results, the sadness and tiredness with each failed attempt and the joy of each successful experiment.

Abstract

Circulating tumour cells (CTC) can be found in blood of cancer patients. A high number of CTC in blood indicates a poor prognosis. These CTC can have an epithelial like, more adhesive phenotype, can be more motile, like cells with a mesenchymal phenotype, or have a hybrid state. The state of cells depends on the degree of epithelial-to-mesenchymal transition (EMT) they underwent. Cells express different markers, depending on their EMT state. The CellSearch system isolates CTC based on EpCAM expression, which are subsequently identified by the expression of cytokeratin. Both of these markers are indicative of an epithelial phenotype and are not expressed in mesenchymal cells. Cells that do not express these markers are either not isolated or not detected by the CellSearch system.

The goal of this project is to identify markers that can be used next to EpCAM and cytokeratin for the identification of CTC subpopulations. Therefore, immunostaining of PSMA, E-cadherin, N-cadherin, cadherin-11, SLUG and EGFR was tested on cancer cell lines and blood cells. For low abundance proteins, the amplification methods proximity ligation assay (PLA) and tyramide signal amplification (TSA) were developed and applied. To determine the cancerous origin of EpCAM negative cancer cells in filtered blood of the CellSearch system, microRNA-21 detection with fluorescent *in situ* hybridisation and TSA was investigated.

After optimisation of the staining protocol, cancer cells could be discriminated from white blood cells with 2 µL anti-PSMA per 100 µL cell suspension, 2 µg/mL E-cadherin or 2 µg/mL cadherin-11 staining. Even though the staining protocol for N-cadherin was optimised to 2 µg/mL it could only be detected in cell-cell contact areas of adhering cells. Spiked tumour cells and white blood cells could not be discriminated based on used anti-SLUG and anti-EGFR staining.

With PLA and TSA low abundance proteins could be detected. Compared to TSA PLA showed better spatial resolution, enabling single molecule quantification.

MicroRNA-21 expression could neither be detected in cells nor when hybridising the anti-microRNA-21 probe against the amplified microRNA-21 gene.

It is concluded that suitable markers for identification of CTC are epithelial marker E-cadherin, mesenchymal marker cadherin-11 and PSMA which is expressed by CTC in different phenotypic states and that low abundance proteins are detected with signal amplification.

Keywords: CTC, EMT, EpCAM, MET, microRNA-21, PLA, TSA, tumour markers

Contents

List of Abbreviations

APC	allophycocyanin
ATCC	American type culture collection
BSA	bovine serum albumin
CTC	circulating tumour cells
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
FBS	fetal bovine serum
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
HRP	horseradish peroxidase
LNA	locked nucleic acid
MCBP	Medical Cell BioPhysics
MET	mesenchymal-to-epithelial transition
microRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin-chlorophyll-protein complex
PLA	proximity ligation assay
PSMA	prostate specific membrane antigen
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RT-PCR	real time polymerase chain reaction
SLUG	SNAI2 zinc finger protein
SSC	saline-sodium citrate
Tm	melting temperature
TSA	tyramide signal amplification

1 Introduction

A neoplasm, from Greek *neo* "new" and *plasma* "thing formed", is the result of the autonomous growth of cells in a specific part of the body. The cancerous cells reproduce uncontrollably and are able to invade locally, causing damage to surrounding tissues, and spread to other areas. [1] The migration of malignant cells is called metastasising and accounts for more than 90% of cancer-related deaths [1–3]. In 2015, 8.8 million people died from cancer in the world. [4] Specific proteins of malignant neoplasms, also called tumour markers, can be detected in the cells themselves or in body fluids.

In a minimally invasive way, circulating tumour biomarkers, such as circulating tumour cells (CTC) from which metastases originate, can be obtained from blood. Biomarkers give information for the selection of a suitable therapy, by presence or absence of targets for targeted therapy, and information about patient prognosis and tumour progression. [1,5–8] CTC have a half-life time in blood of 1.0 to 2.4 h [9], which enables dynamic monitoring in time. However, CTC are rare and using the CellSearch system currently only CTC are captured that express epithelial cell adhesion molecule (EpCAM) [6, 8].

1.1 Origin of circulating tumour cells

Epithelial cells and malignant epithelial cells are non-motile and encased via cell-cell tight junctions. To be able to invade and colonise another part of the body, the cells must undergo a transformation. The steps that primary tumour cells undergo during metastasis, called epithelial-to-mesenchymal transition (EMT), are thought to be as described below.

First, a cell adapts to a phenotype that permits enhanced motility, detaches from its neighbouring cells in the primary tumour and breaks through the basement membrane by dissolving it. This makes it a malignant tumour. Invasion of these malignant cells then requires the passage through the extracellular matrix (ECM). Proteolytic enzymes, such as matrix metalloproteinases, are expressed on invadopodia by invading cells, leading to degradation of the ECM. Invadopodia also help navigating sensing chemoattractive molecules and through exploration of cell-cell and cell-matrix adhesions.

At some point, the cell attaches to a blood vessel wall, mediated by interactions of expressed adhesion molecules. Proteolytic enzymes are released, breaking proteins into shorter fragments and damaging the blood vessel line-up, and the cancer cell enters into the blood or lymphatic circulation. [1] The lymphatic circulation is out of the scope of the project and will not be discussed. The cells, now called CTC, are transported and can undergo interactions with blood components, such as platelets which protect the cells from the immune system. Next, the CTC arrive at the secondary site and exit the bloodstream. They are able to do so, by inducing cell death or retraction of endothelial cells lining up the blood vessel wall. Last, the cells undergo reverse EMT, called mesenchymal-to-epithelial transition (MET). [1,2,11] After migrating to their new spot, the metastatic site, they die, enter a dormant state or are able to continue with proliferation and form micrometastases. [1, 2, 12] To grow beyond about 2 mm in diameter [1], additional nutrients and oxygen supply are needed. Therefore, tumours stimulate tumour-associated new blood vessel formation to enable them to grow into macrometastases or metastatic tumours. [1, 2, 13] To what degree this EMT process takes place in released tumour cells is still unclear, and that CTC co-express both epithelial and mesenchymal markers cannot be excluded. [11] CTC are likely to balance between the epithelial and mesenchymal phenotype, rather than express only one of both. [11, 14]

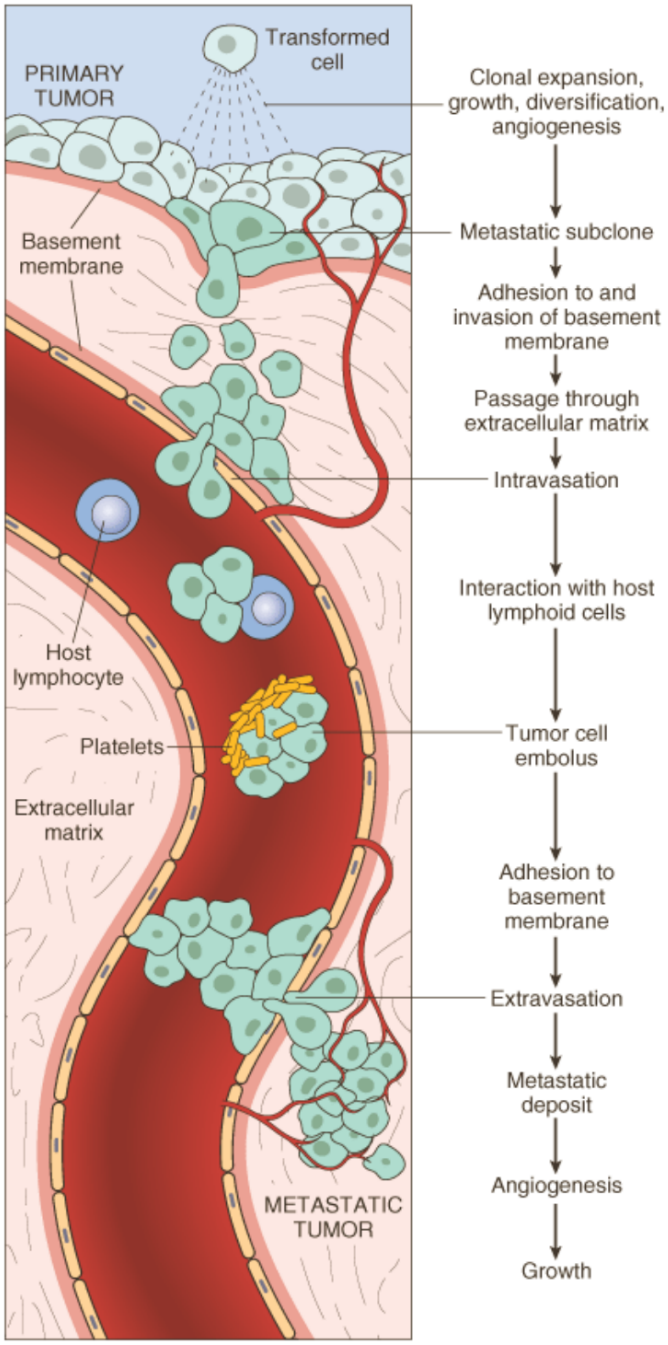


Figure 1: Tumor cell progression. [10] Epithelial cells on top are transformed and have lost their contact with the other cells and are able to break through the basement membrane. They gain a more mesenchymal like phenotype, undergoing partly or fully completed EMT. Motility of these transformed cells is increased and after migrating through the extracellular matrix, they can enter the blood stream. The now called CTC can interact with blood components and the blood will transport these cells to a secondary site. Cells will leave the blood stream and form a secondary, metastatic tumour, undergoing MET. [1–3]

1.2 Detection of circulating tumour cells

When a blood sample is drawn from a cancer patient, CTC can be found. The presence of these CTC is an indication of a worse prognosis for the patient. [15–17] Detection of CTC is difficult, since they are very rare in the blood, with 1 to 10 CTC per millilitre of blood of patients with metastatic cancer. [17–19] Per millilitre of blood, there are around 7 million white blood cells and 5 billion red blood cells. [20]

For the isolation of CTC from peripheral blood, the by the food and drug administration approved CellSearch system (Janssen Diagnostics, LLC, Raritan, NJ, United States) can be used. CTC are defined as cells with a nucleus; expressing cytokeratins which demonstrate epithelial origin; have an absence of CD45, indicating the cell is not of hematopoietic origin; and are often larger cells with irregular shape or subcellular morphology. CTC enrichment is performed using EpCAM targeted immunomagnetic selection. [5, 17]

Due to the process of EMT, there should be found no or only a small number of CTC of epithelial origin by the CellSearch system because most cells would be in a mesenchymal state. [21] So far however no studies have shown consistent high numbers of CTC missed by the CellSearch system due to their mesenchymal state. This shows that CTC probably balance between the epithelial and mesenchymal phenotype [11, 14]. As a result, there are CTC that express lower EpCAM [21–23], or lost their EpCAM expression and are therefore missed by CellSearch. [6, 21]

To be able to also enrich the CTC with low or no expression of EpCAM, other antigens that are expressed by these CTC, and not by other blood components, are needed.

1.3 Epithelial-to-mesenchymal transition markers

The process of EMT is visualised in figure 2. The cuboidal epithelial CTC expresses markers such as E-cadherin, EpCAM and cytokeratins. The mesenchymal CTC has lost some markers and gained other, like vimentin, N-cadherin and cadherin-11.

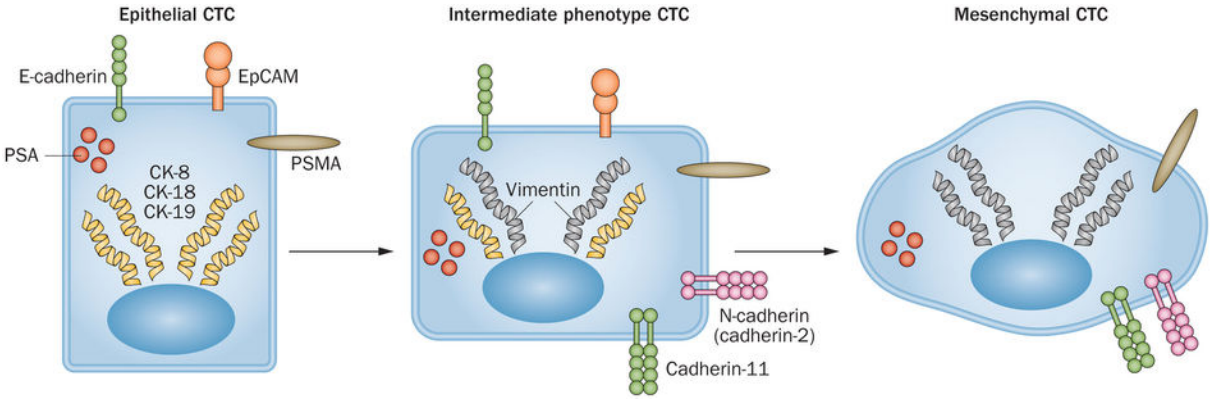


Figure 2: Epithelial-to-mesenchymal transition. [24] The cuboidal cell on the left represents a CTC with an epithelial phenotype. Epithelial markers such as EpCAM and E-cadherin are expressed. When transforming to a more mesenchymal phenotype, some epithelial markers are lost and other markers are gained like vimentin and cadherin-11, which represent a mesenchymal phenotype. [24]

To be able to catch EpCAM low or negative cells, the following antigens that are potentially expressed on CTC are investigated: N-cadherin, E-cadherin, cadherin-11, epidermal growth factor receptor (EGFR), SNAI2 zinc finger protein (SLUG), prostate specific membrane antigen (PSMA) and EpCAM. See also table 1. This panel of antibodies can be used for the three phenotypes, being the epithelial phenotype, mesenchymal phenotype and the phenotype in between those two extremes.

1.3.1 EpCAM

Epithelial cell adhesion molecule, EpCAM, is expressed on the outside of most normal epithelial cells. The antigen is a homotypic calcium-independent cell adhesion molecule. [25, 26] During EMT, EpCAM is downregulated. [24]

1.3.2 Cytokeratins

Cytokeratins are intermediate filaments proteins and are found inside the cell. [24, 25] There are about 20 cytokeratins known. They are responsible for the structural integrity of epithelial cells [25]. Which cytokeratins are expressed differs per cancer type. [25,27,28]

1.3.3 PSMA

Prostate specific membrane antigen, PSMA, is expressed in epithelial as well as in mesenchymal phenotypic cells and is located on the cell membrane. [24,29] PSMA is expressed in most prostate cancers and upregulation is found to correlate with increased aggressiveness. This suggests that PSMA plays a role in prostate cancer progression. [29, 30]

1.3.4 E-cadherin

Cadherins are a family of cell-cell adhesion molecules that are calcium dependent transmembrane glycoproteins. E-cadherin is expressed on the surface of epithelial cells. In most carcinomas, malignant epithelial tumours, the expression of E-cadherin is reduced or lost. Due to this loss, individual malignant cells are allowed to leave the primary tumour and metastasise. [31]

1.3.5 Cadherin-11

Expression of cadherin-11 is associated with EMT, mesenchymal tissue formation and tumour progression. Cadherin-11 expression is found only in highly-invasive and poorly differentiated cancer cells and in stromal cells and osteoblasts that normally express cadherin-11. Chu et al. (2008) could only find cadherin-11 transcripts in prostate cancer cells that were derived from human bone metastases (PC3), but not in metastasis that were derived from lymph nodes (LNCaP) [32]. It is localised on the cell membrane. [33–35]

1.3.6 N-cadherin

N-cadherin is a calcium-dependent cell adhesion molecule, found on the cell membrane. [25] It provides a mechanism for transendothelial migration, when adhering to the blood vessel wall. The intracellular connection between two adjacent cells of the blood vessel wall fails due to upregulation of biological pathways, so that the cancer cell is able to slip through. [36]

Table 1: Antigen expression according to literature. No expression is indicated with a (-) and positive expression is shown qualitatively with (+/-) representing barely detectable up to (++) representing high marker expression. References are given in parentheses.

	PC3		MCF-7		LNCaP	
N-cad	+/-	[25]	-	[25]	-	[43]
E-cad	-	[25]	++	[25, 44]	++	[45]
Cadherin-11	+	[25, 46]	-	[25, 33]	-	[32]
EGFR	+/-	[25, 47]	+/-	[25, 48]	+/-	[25, 47]
SLUG	+	[49]	-	[25, 50]	+/-	[49]
PSMA	-	[51]	-	[52]	+	[51]
EpCAM	+/-	[25]	++	[25]	++	[53]
Cytokeratins	+	[25, 27]	+	[25, 28]	+	[25, 27, 54]

1.3.7 SLUG

SLUG, zinc finger protein SNAI2, is a transcription factor, found inside the cell. SLUG acts as a master regulator and induces EMT. SLUG has antiapoptotic activity and represses epithelial markers such as E-cadherin, whereas it upregulates mesenchymal markers such as fibronectin and vimentin. [25, 37–41]

1.3.8 EGFR

Epidermal growth factor receptor is found on the cell membrane and binds to epidermal growth factor. Binding will induce a signalling cascade, resulting in cell proliferation. [25] Autocrine activation of EGFR leads to EMT. [42]

1.4 Signal amplification

In a cell not all proteins are expressed at the same level; some are high abundant, some low abundant. Markers with a low expression are difficult to detect using standard fluorescent detection methods. A signal amplification method can be used to improve the detection of these markers that can be missed using immunostaining without amplification due to lack of detection sensitivity. Signal amplification strategies can be divided into two types, being enzyme labelling and macrofluorophore labelling techniques.

Macrofluorophores are collections of fluorophores ranging from tens [55] to millions [56]. These collections are attached to a scaffold or are incorporated in it. This scaffold is coupled to a target-specific reagent such as an antibody. Opposite to enzyme labelling techniques, they have no time-dependent signal development, but are more prone to non-specific binding [57].

For enzyme labelling techniques, an enzyme is linked to a target-specific reagent, using direct conjugation or indirect conjugation trough a secondary complex. This enzyme provides multiple copies of a fluorophore. Signal levels are increased in comparison to dye-labelled reagents. One of these enzymes is horseradish peroxidase (HRP), which is used in this project. Applications in immunocytochemistry require that the product of the enzyme reaction is in the vicinity of the enzyme conjugate, so information about the spatial distribution of the target is obtained. Enzyme reactions are time dependent. Therefore, control of timing is crucial to obtain reproducible results.

In case of nucleic acids, amplification of the target is possible through polymerase chain reaction. The enzyme polymerase synthesises chains of nucleic acids. Polymerase is used in proximity ligation assay during the rolling circle amplification step. [58]

1.4.1 Tyramide signal amplification

Another technique to amplify signal is tyramide signal amplification (TSA), see figure 3. To target the antigen of interest, a primary antibody is used. Against this primary antibody, a HRP-labelled secondary antibody is targeted. Then a dye, here a tyramide derivative, and hydrogen peroxide is added. HRP activates multiple copies of the tyramide derivative. The resulting highly reactive tyramide radicals covalently bind to residues in the vicinity of the HRP-target interaction site. Since they are highly reactive short-lived tyramide radicals, there is minimal diffusion related loss of signal. [59–61]

1.4.2 Proximity ligation assay

Proximity ligation assay (PLA), see figure 4, is a technique which enables the specific detection and quantification of low abundance proteins and biomarkers. PLA is based on the proximal binding of two probes with deoxyribonucleic acid (DNA) strands targeted against the protein of interest, joined by ligation, resulting in the formation of a surrogate marker. Targets can be different epitopes of the same protein, or two proteins situated in close proximity. The enzymatic reactions ligation and amplification to modify DNA can be used to tweak the surrogate markers in a proximity-dependent manner. [62, 63] Applications of PLA are detection and quantification of protein-protein interactions, post-translational modifications and low expression proteins. Protein-protein interactions can be visualised using two different primary antibodies against two epitopes localised on two different proteins. Signal is generated when the target proteins have a maximum distance of 40 nm from each other. Post-translational modifications can be visualised

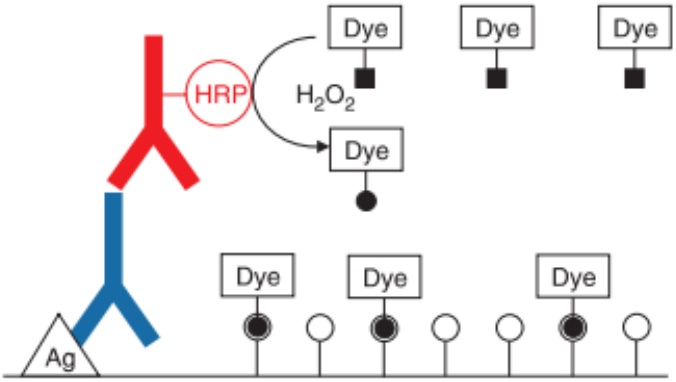


Figure 3: TSA labeling is a combination of three processes. First, a probe binds to its target via immunoaffinity in the case of proteins or hybridization in the case of nucleic acids. A secondary antibody that is HRP-labelled then targets the primary antibody. In the second phase, activation of multiple tyramide derivatives by HRP takes place. Thirdly, the resulting highly reactive, short-lived tyramide radicals covalently bind to residues in the vicinity of the HRP molecule. [59, 60]

using one antibody against the protein and one against its modification of interest. If the modification is present on the protein they are in proximity and both antibodies bind, so signal is generated. PLA enables detection of a single event using target specific antibodies. [64] In this project PLA is used to detect low abundance proteins.

For the first step of targeting, primary antibody-epitope interactions are used. Against the primary antibody, secondary antibodies are targeted. To the secondary antibodies, called PLUS and MINUS probe, short single stranded DNA oligonucleotides have been attached. Two circle-forming DNA oligonucleotides hybridise with the PLUS and MINUS probe. If they are in close proximity (<40nm) [64], ligase catalyses the joining of their endings and a DNA circle is formed. This DNA circle is amplified by polymerase, an enzyme that synthesises long chains of nucleic acids. This process is also known as rolling circle amplification. Fluorescently labelled oligonucleotide probes bind to the amplified repetitive sequences of DNA. With fluorescent microscopy, they are seen as one single dot, although they are composed of around 1000 bound fluorescent probes. [62, 64, 65] In this project, one primary antibody is used for single recognition, but it is also possible to use two types of primary antibodies targeted against two epitopes localised on the same protein, called double recognition. Double recognition increases the specificity and single recognition the sensitivity. [64]

1.5 MicroRNA-21

The increased sensitivity of TSA can also be helpful for the detection of low-abundance micro ribonucleic acid (microRNA) by fluorescence *in situ* hybridization (FISH). [60] Cells with cytokeratin expression detected on filtered blood from cancer patients are expected to be CTC. There is a possibility however, that these cells are normal epithelial cells. Whether these cells are normal or tumorous, can be demonstrated with the detection of cancer specific microRNA using FISH. A probe with a fluorescent marker can

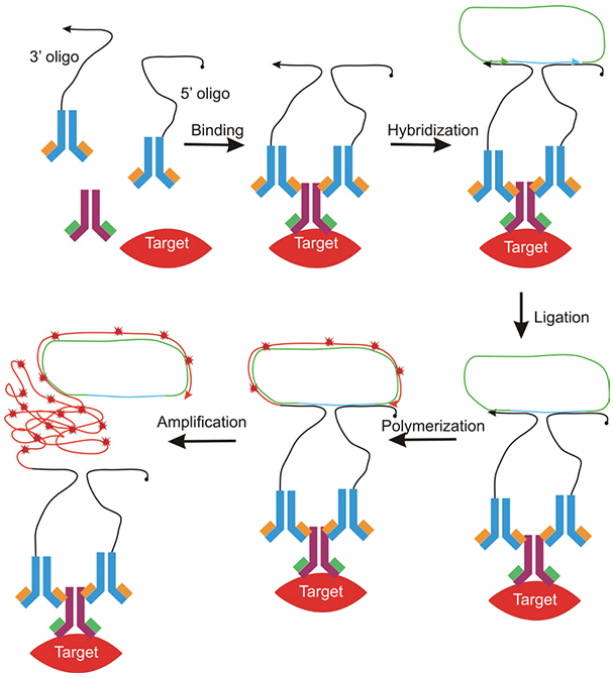


Figure 4: PLA principle. [66] After incubation with the primary antibody against the target, the PLUS and MINUS probes with 3' and 5' oligo are targeted against the primary antibody. If the probes are in close proximity (<40nm), two circle-forming DNA oligonucleotides bind to the probes. Ligase then catalyses the joining of the endings to form a complete DNA circle. Subsequently, rolling circle amplification takes place. Thereafter, fluorescent probes hybridise with the amplified DNA. Under the microscope, the result is seen as a single dot. [64, 66]

be hybridised against microRNA and detected with fluorescence microscopy, quantitative polymerase chain reaction and digital droplet polymerase chain reaction. MicroRNAs are short (20-24 nucleotides) non-coding ribonucleic acids (RNAs) that are involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of messenger ribonucleic acids (mRNAs). They can be protein-coding or non-coding. MicroRNAs are important in cellular processes such as proliferation, differentiation and apoptosis. [67–69]

In this project the focus is on microRNA-21. It is located in the cytoplasm [70] and it has been found to be elevated in different types of solid tumours. [37, 71] MicroRNA-21 is a strong antiapoptotic and prosurvival microRNA. [67, 71, 72] In a meta-analysis about the diagnostic and prognostic value of microRNA-21 in colorectal cancer it was found that higher expression of microRNA-21 correlated to inferior overall and disease-free survival. [73] The microRNA is incorporated into a RNA-induced silencing complex. A RNA-induced silencing complex consists of multiple proteins with a mass of approximately 500 kDa [74]. In this complex microRNA acts as template to recognise target mRNA through imperfect base pairing. This often results in destabilization, cleavage and translational inhibition of the target mRNA. This process is called RNA interference. [75, 76] The concentration of active RNA-induced silencing complexes is estimated to be 3 to 5 nM per cell. [77, 78] An elevated level of microRNA-21 expression is seen in NCI-H1975 and MCF-7 cells, but not in NCI-H1650 cells. [79, 80]

1.6 Aim

CTC give information about the cancer patient status and ideally all CTC present are detected. Unfortunately, CTC are very rare in blood. CTC are isolated using EpCAM, causing EpCAM negative CTC to be lost, which are most likely to be cancer cells that undergo EMT.

The aim of the project is to determine the presence of previously described EMT related markers on CTC and identify EpCAM negative CTC. Tests to optimise staining are performed using monoclonal antibodies on different cancer cell lines. The hypothesis is that more circulating tumour cells are detected using EpCAM, cytokeratins and one or multiple of the investigated EMT-markers, compared to using only EpCAM and cytokeratins on the CellSearch system.

Two signal amplification techniques are investigated: TSA and PLA. Signal amplification is used to improve detection of low abundance markers.

TSA is used to detect microRNA in CTC. Cells that express cytokeratin are found in filtered blood of the CellSearch system, and might or might not be CTC. A distinction can be made between CTC and normal epithelial cells based on the detection of cancer specific microRNA.

2 Materials and methods

2.1 Cell lines

Human prostate carcinoma cell line PC3 (American type culture collection (ATCC) CRL-1435, 17.7 µm in diameter, 10,443 EpCAM molecules per cell [81]) was derived from a metastatic site in the hip bone. The genetically modified prostate cancer cell lines PC3αN cat 5.2, which has a very low cadherin-11 expression, and PC3αN cat 92.1, which has a high cadherin-11 expression, were obtained from Radboud University Medical Centre Nijmegen, the Netherlands.

Human prostate carcinoma cell line LNCaP (ATCC CRL-1740, 16.3 µm in diameter, 637,278 EpCAM molecules per cell [81]) was derived from a metastatic site in the left supraclavicular lymph node.

Human bronchoalveolar carcinoma cell line NCI-H1650 (ATCC CRL-5883, 12.0 µm in diameter, 135 EpCAM molecules per cell [81]) was derived from a metastatic site in the pleura and was chosen for its low EpCAM expression and non-elevated level of microRNA-21 expression.

Human lung adenocarcinoma cell line NCI-H1975 (ATCC CRL-5908, 17.6 µm in diameter, 397,038 EpCAM molecules per cell [81]) was derived from a non-small cell lung cancer and was chosen for its elevated level of microRNA-21 expression.

Human breast adenocarcinoma cell line MCF-7 (ATCC HTB-22, 16.3 µm in diameter, 880,189 EpCAM molecules per cell [81]) was derived from a metastatic site in the pleura.

Human cervical adenocarcinoma cell line HeLa (ATCC CCL-2) was chosen for its N-cadherin expression.

Human umbilical vein endothelial cell line HUVEC (ATCC PCS-100-010) was obtained from the group Molecular Nanofabrication, University Twente, and tested for its N-cadherin expression.

2.2 Cell culture

Prostate cancer cell lines PC3 and LNCaP and lung cancer cell line NCI-H1650 (Medical Cell BioPhysics (MCBP)) were cultured in RPMI-1640 medium (Lonza, Basel, Switzerland, cat: BE12-702F, lot: 6MB165) supplemented with 10% fetal bovine serum (FBS) (Sigma, Saint Louis, United States of America, cat: F7524, lot: 124M3337) and 1% penicillin/streptomycin (10 000 u/ml, Lonza, lot: 4MB132) at 37 °C and 5% CO_2.

The genetically modified prostate cancer cell lines PC3αN cat 5.2 and PC3αN cat 92.1 were cultured in RPMI-1640 (Lonza) supplemented with 10% FBS (Sigma), 1% penicillin/streptomycin (10 000 u/ml, Lonza) and 0.4% geneticin (Gibco, Waltham, United States of America, cat: 10131-035, lot: 1852838) at 37 °C and 5% CO_2.

The breast cancer cell line MCF-7 was cultured in DMEM (Lonza, cat: BE12-604F, lot: 6MB175) supplemented with 10% FBS (Sigma) and 1% penicillin/streptomycin (10 000 u/ml, Lonza) at 37 °C and 5% CO_2.

The cervical cancer cell line HeLa was cultured in MEME (Sigma) supplemented with 10% FBS (Sigma), 1% penicillin/streptomycin (10 000 u/ml, Lonza) and 1% L-glutamine (200 mM, Lonza) at 37 °C and 5% CO_2.

Cells were harvested with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco). For N-cadherin and cadherin-11 expression experiments however, cells were harvested both with 0.05% trypsin-EDTA and 15 mM EDTA. For fixation, cells were resuspended in 1% formaldehyde (Sigma) in 1X phosphate buffered saline (PBS) (Sigma)

Table 2: Immunofluorescent staining

Antibody	Clone	Brand	Cat	Lot	Concentration
EpCAM	VU-1D9	Sigma-Aldrich	SAB4700424	527713	2 µl per 100 µL
E-cadherin	67A4	BioLegend	324107	B167426	2 µg/ml
N-cadherin	8c11	BioLegend	350809	B184593	2 µg/ml
Cadherin-11	16.a.c.15	Nijmegen			2 µg/ml
Cadherin-11	16G5	BioLegend	368702	B206555	2 µg/ml
GaM IgG-PE		Life Technologies	P21129	1828010	2 µg/ml
EGFR	REA439	Miltenyi Biotec	130107717	5150629283	5 µl per 100 µL
EGFR	REA439	Miltenyi Biotec	130107716	5150629282	5 µl per 100 µL
PSMA	REA408	Miltenyi Biotec	130106612	5150629236	2 µl per 100 µL
SLUG	REA404	Miltenyi Biotec	130106186	5150629281	5 µl per 100 µL
CD45	H130	Life Technologies	MHCD4531	1749876A	4 µl per 100 µL
CD16	3G8	BioLegend	302030	B235385	2 µg/mL

for 15 minutes at room temperature. Afterwards, cells were pelleted and resuspended in 1% bovine serum albumin (BSA) (Sigma) in 1X PBS (Sigma), stored at 4 °C and used within two weeks.

2.3 Blood lysis

Whole EDTA blood from healthy donors, who gave informed consent, was lysed to obtain white blood cells. To destroy red blood cells, per 1 ml of blood 13 ml ammonium chloride lysing solution was used (15 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.25) under gently mixing at room temperature until a clear solution was obtained. For fixation, cells were pelleted and resuspended in 1% formaldehyde (Sigma) in 1X PBS (Sigma) for 15 minutes at room temperature. Cells were pelleted and resuspended in 1% BSA (Sigma) in 1X PBS (Sigma), stored at 4 °C and used within two days.

2.4 Immunofluorescent staining

Antibodies and concentrations that were used are given in table 2. For intracellular markers, cells were permeabilised using 1X PBS (Sigma) with 1% BSA (Sigma) and 0.05% saponin (Sigma). Cells incubated 30 minutes at 37 °C with a primary antibody. When a secondary antibody was used, cells were washed in 1% BSA in 1X PBS, pelleted and aspirated, before incubating 30 minutes at 37 °C with the secondary antibody. After staining with primary antibody, and eventually secondary antibody, cells were resuspended 1% BSA in 1X PBS. Then, nuclei were stained with 4 µg/ml Hoechst 33342 (Molecular Probes, Waltham, United States of America) for 15 minutes at room temperature and white blood cells with either anti-CD45 or anti-CD45 and anti-CD16. The extensive protocol for staining in solution is given in appendix A.

2.5 Tyramide signal amplification

TSA was performed using the Alexa Fluor 488 Tyramide SuperBoost Kit, goat anti-mouse IgG, purchased at Invitrogen (Eugene, United States of America, cat: B40941, lot: 1863934). It contains blocking buffer (10% goat serum), poly-HRP-conjugated goat

anti-mouse secondary antibody (1X), Alexa Fluor tyramide reagent, hydrogen peroxide (stabilised 3% solution), reaction buffer (20X), reaction stop solution and dimethylsulfoxide (DMSO). Along with poly-HRP-conjugated goat anti-mouse secondary antibody, rabbit anti-FITC:HRP was used (1 mg/ml, BioRad, Hercules, United States of America, cat: 4510-7864, lot: 161212).

TSA was performed on cells in solution and on cover slips (Menzel-Gläser, ThermoFisher). When using cells on cover slips, cells were grown for two days at a high cell concentration and fixed when attached to the cover slips. For TSA in solution, also fixed cells were used. A step-by-step protocol is given in appendix B. After fixation, samples were incubated with 3% H_2O_2 for 60 minutes at room temperature to quench endogenous peroxidase activity. Then, cells were rinsed three times with 1X PBS and incubated with blocking buffer (10% goat serum, Invitrogen) at 37 °C. Then, primary antibodies were used with mouse as host or labelled with fluorescein isothiocyanate (FITC), depending on the horseradish peroxidase (HRP) used in the next step. After incubation for 30 minutes at 37 °C, samples were rinsed once with 1X PBS and incubated either with anti-mouse:HRP (goat anti-mouse IgG poly HRP conjugate, Invitrogen) or 2 µg/ml anti-FITC:HRP (rabbit anti FITC:HRP, Bio-Rad) for 30 minutes at 37 °C. Samples were rinsed three times with 1X PBS for 10 minutes at room temperature. Meanwhile, 1X reaction buffer (Invitrogen), 1:11 reaction stop reagent working solution (Invitrogen), 100X H_2O_2 solution (Invitrogen) and tyramide working solution (Alexa Fluor 488 tyramide, Invitrogen) were prepared according to the protocol of Invitrogen. Samples were incubated with tyramide working solution for 15 minutes at room temperature, whereafter reaction stop reagent working solution was added to stop the HRP reaction. Samples were rinsed three times with 1X PBS. 4 µg/ml Hoechst 33342 (Molecular Probes) was added to the cells in solution. Cover slips were flipped on a drop of mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Duolink). After 15 minutes incubation at room temperature, samples were analysed under the microscope.

2.6 Proximity ligation assay

PLA was performed using Duolink *in situ* PLA probe anti-mouse PLUS (Olink Bioscience, Uppsala, Sweden, cat: DUO92001-30RXN, lot: A63409/1) and Duolink *in situ* PLA probe anti-mouse MINUS (cat: DUO92004-30RXN, lot: A62707/2). Both products are comprised with 5X PLA probe anti-mouse PLUS, respectively MINUS, 1X blocking solution and 1X antibody diluent.

PLA was performed on cells in solution and on cover slips (Menzel-Gläser, ThermoFisher). When using cells on cover slips, cells were grown for two days at a high cell concentration and fixed when attached to the cover slips. For PLA in solution, also fixed cells were used. Unless stated otherwise, reagents were obtained from Duolink. A step-by-step protocol is given in appendix C. After fixation, samples were washed twice with 1X PBS (Sigma) for 2 minutes and incubated with 1X blocking solution at 37 °C for 30 minutes. Primary antibody EpCAM (Veridex, clone VU1D9, unconjugated) was diluted in 1X antibody diluent to a concentration of 5 µg/ml and added to the samples, which incubated overnight at 4 °C. Samples were rinsed twice with 1X wash buffer A for 5 minutes. The two PLA probes PLUS and MINUS were diluted 1:5 in 1X antibody diluent and samples were incubated with this mixture for 60 minutes at 37 °C. Samples were washed twice in 1X wash buffer A for 5 minutes under gentle agitation. For ligation, ligation stock was diluted 1:5 in high purity water and ligase was added to this solution

to a dilution of 1:40. Samples incubated with this mixture for 30 minutes at 37 °C and were then washed twice with wash buffer A for 2 minutes under gentle agitation. For amplification, amplification stock was diluted 1:5 and polymerase 1:80 in high purity water and added to the samples to incubate for 100 minutes at 37 °C. Samples were washed twice in 1X wash buffer B for 10 minutes and subsequently once in 0.01X wash buffer for 1 minute. 4 µg/ml Hoechst 33342 (Molecular Probes) was added to the cells in solution. Cover slips were flipped on a drop of mounting medium with DAPI. After 15 minutes incubation at room temperature, samples were analysed under the microscope.

2.7 MicroRNA-21 detection

For microRNA-21 detection two methods were used, described below.

In situ hybridisation was performed using a specific anti-microRNA-21 5'-fluorescein labelled miRCURY locked nucleic acid (LNA) probe from Exiqon (Vedbaek, Denmark, cat: 610472-310). The probe sequence is AGCCCATCGACTGGTGTT. The effective melting temperature (Tm) was calculated using formula

$$Tm = 81.5 + 16.6 * log\ \mathrm{M}[Na^+] + 0.41 * \%GC - 0.72 * \%formamide \quad (1)$$

with 56% the guanine cytosine (GC) content of the probe sequence and 50% formamide used in the hybridisation buffers. Calculated Tm with different saline-sodium citrate (SSC) concentrations used are given in table 3.

Table 3: Calculated effective melting temperature

SSC	M [$\mathbf{Na^+}$]	**Tm in** °C
1X	0.165	55.3
2X	0.33	60.3
3X	0.495	63.2
4X	0.66	65.3
5X	0.825	66.9

The first method was based on the article from Gasch *et al.* [82]. NCI-H1975, NCI-H1650 and MCF-7 cells were grown on cover slips, fixed and permeabilised with 0.1% Triton X-100 in 1X PBS for 10 minutes at room temperature with washing in between the steps. Endogenous peroxidase was quenched with 3% H_2O_2 for 60 minutes at room temperature. Samples were washed three times with 1X PBS before blocking in hybridisation buffer at 50 °C for 10 minutes. Different hybridisation buffers were used. Hybridisation buffers contained 50% formamide, 0.5 mg/ml DNA from fish sperm (Sigma, cat:74782, lot: 1343783), and 1X, 2X, 3X, 4X or 5X SSC. Following blocking with hybridisation buffer the buffer was replaced with hybridisation buffer containing 40 nM anti-microRNA-21 probe and the cover slips left to incubate at 50 °C for 60 minutes. Drying of cover slips was prevented. The cover slips were then subjected to five stringency wash steps of 5 minutes at 50 °C: once with 5X SSC, twice with 1X SSC and twice with 0.2X SSC, before washing in 0.2X SSC at room temperature for 5 minutes. Samples were then washed twice with 1X PBS for 10 minutes at room temperature before blocking with blocking buffer (10% goat serum, Invitrogen) for 30 minutes at 37 °C. Blocking buffer was removed before adding 2 µg/ml anti-FITC:HRP. Cover slips left to incubate at 37 °C for 30 minutes. Samples were washed three times for 10 minutes with 1X PBS at room temperature. Meanwhile,

1X reaction buffer (Invitrogen), 1:11 reaction stop reagent working solution (Invitrogen), 100X H_2O_2 solution (Invitrogen) and tyramide working solution (Alexa Fluor 488 tyramide, Invitrogen) were prepared according to the protocol of Invitrogen. Samples were incubated with tyramide working solution for 15 minutes at room temperature, whereafter reaction stop reagent working solution was added to stop the HRP reaction. Samples were rinsed three times with 1X PBS. Cover slips were flipped on a drop of mounting medium with DAPI (Duolink). After 15 minutes incubation at room temperature, samples were analysed under the microscope.

The second method used was detection of the microRNA-21 gene with polymerase chain reaction (PCR). Isolated genomic DNA was obtained from MCBP, University of Twente, the Netherlands. Gene expression was analysed by real time polymerase chain reaction (RT-PCR) using the CFX96 Touch Real-Time PCR Detection System with C1000 Touch ThermalCycler and CFX Manager 3.1 software from BioRad. Primers for analysis of microRNA-21 gene were developed with the Integrated DNA Technologies design tool. Three assay sets consisting of a forward and reverse primer were ordered. They were designed to have around 50% GC content, a primer length of 20 nucleotides, a 62 °C melting temperature and around 200 amplicon length with the target sequence halfway. The three primer sets were:

1. Forward: TTGCCTACCATCGTGACATC
   Reverse: CAGACAGAAGGACCAGAGTTTC

2. Forward: CACCTTGTCGGGTAGCTTATC
   Reverse: AATCCTCCCTCCATACTGCT

3. Forward: GTGACATCTCCATGGCTGTA
   Reverse: CTAAGTGCCACCAGACAGAA

PCR was performed in 25 µL with the following RT-PCR program: 1 cycle of 95 °C for 3 minutes and 40 cycles of 95 °C for 30 s, a temperature gradient of 56 to 64 °C for 30 s and 72 °C for 30 s for amplification, with a final elongation step of 72 °C for 10 minutes. As references oligos gChr4SCARB2f in combination with gChr4SCARB2r (amplicon size: 197 basepairs) and gChr1SCAMP3f in combination with gChr1SCAMP3r (amplicon size: 301 basepairs) were used. Reference material was collected and served as negative control. The amplified microRNA-21 gene material was collected and served as positive control. Amplified DNA was purified with the Qiagen DNeasy blood and tissue kit (Venlo, the Netherlands, cat: 69504, lot: 157033522) and eluted in high purity water. Yield was measured with the Invitrogen Qubit 2.0 Fluorometer. Obtained yields were 2.90 ng/µL for the negative control and 1.82 ng/mL for the positive control. Eluted DNA was stored at −30 °C until use on the next day. It was denatured at 96 °C and placed on ice water directly afterwards. 1 µL of the positive and 1 µL of the negative control were spotted on a polyvinylidene fluoride (PVDF) membrane (BioRad, cat: 1620260, lot: 20170214). Two hybridisation buffers were used: (1) 50% formamide, 0.5 mg/mL fish sperm and 5X SSC and (2) 50% formamide, 1% BSA and 5X SSC. After blocking with hybridisation buffer for 10 minutes at 50 °C, anti-microRNA-21 probe was added to a concentration of 50 nM and left to incubate for 60 minutes at 50 °C. PVDF membranes were placed on a microscopic slide and analysed under the microscope.

2.8 Analysis

Except for microRNA-21 detection with RT-PCR, samples were analysed using fluorescent microscopy and/or flow cytometry.

For fluorescent microscopy, light travels first through an excitation filter, allowing only light of the excitation wavelength to pass with a certain band width. The dichroic mirror reflects the excitation beam onto the sample. Fluorophores on the sample then enter the excited state and emit photons. These photons can pass the dichroic mirror and go via an emission filter to a detector, which can be a camera or the eye. For fluorescent microscopy, the Nikon Eclipse E400 microscope, 40X/0.60 NA sPlanFluor objective, filter cubes DAPI, FITC, phycoerythrin (PE), allophycocyanin (APC) and peridinin-chlorophyll-protein complex (PerCP), Hamamatsu ORCA-Flash 4.0 LT digital CMOS camera C11440-42U, HoKaWo imaging software version 2.6 from Hamamatsu, Icy version 1.9.4.0 and ImageJ 1.51k were used.

For flow cytometry, the BD FACSAria II cell sorter from BD Bioscience and the FACS-Diva software version 8.0.2 were used. Flow cytometry is a laser-based technology that was used for biomarker detection, as depicted in figure 5. It suspends the immunostained cells in a stream of fluid, letting pass one cell at the laser beam at a time. Once a cell passes the laser beam, it scatters light. Forward scatter tells something about the size of the cell, side scatter about the granularity or complexity of the cell and when fluorescently labelled, fluorescent channels detect the light that is emitted by the excited fluorophore.

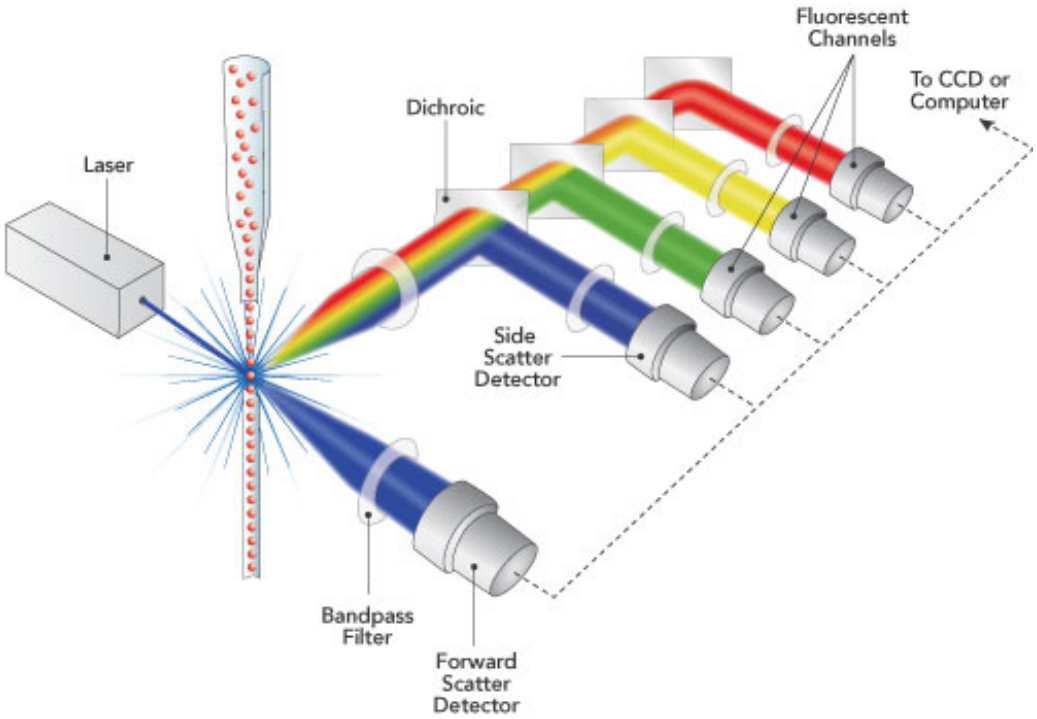


Figure 5: Flow cytometry. Flow cytometry is a laser-based technology that is used for biomarker detection. It suspends the immunostained cells in a stream of fluid, letting pass one cell at the laser beam at a time. Once a cell passes the laser beam, it scatters light. Forward scatter tells something about the size of the cell, side scatter about the granularity or complexity of the cell and when fluorescently labelled, fluorescent channels detect the light that is emitted by the excited fluorophore.

3 Results

The aim of this research was to find epithelial and mesenchymal markers that can be used besides EpCAM, to investigate signal amplification methods to improve detection of low abundance markers and to use TSA for the detection of microRNAs by FISH. Findings are described below.

3.1 Epithelial-to-mesenchymal transition markers

All antibodies were titrated on the highest expressing cell line, before comparing the expression between PC3, MCF-7 and LNCaP cells unless mentioned otherwise. Optimal concentrations were chosen based on the increase of response units in flow cytometry (see table 4) and intensity in microscopic images with increasing concentration, and the ability to distinguish signal from background. More detailed information about antibodies is given in table 2. After determining the optimal concentration per antibody, the antibodies were tested on PC3, MCF-7, LNCaP and white blood cells. Results are described below.

Table 4: Results of the titration tests using several concentrations of antibody for detection. Values are the mean values as derived from flow cytometric analysis. Mean responses of the concentrations which were used in further experiments are given in bold. In brackets are the cell lines which were used for titration of that antibody.

(a)

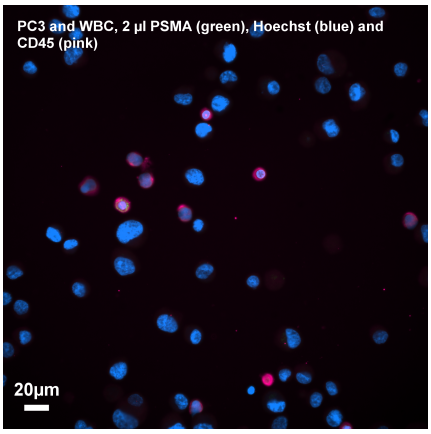
	1 µg/mL	2 µg/mL	4 µg/mL	5 µg/mL
E-cad (LNCaP)	4,346	**5,956**	6,615	-
Cad-11 (PC3αN cat 92.1)	4,639	**5,147**	-	6,587
N-cad (PC3)	86	**97**	110	-

(b)

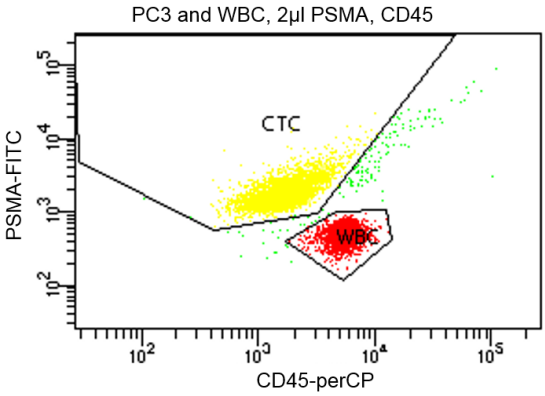
	2 µL per 100 µL	5 µL per 100 µL	10 µL per 100 µL
PSMA (LNCaP)	**10,159**	12,087	14,013
EGFR (PC3)	162	265	332
SLUG (PC3)	782	1,837	2,361

3.1.1 PSMA

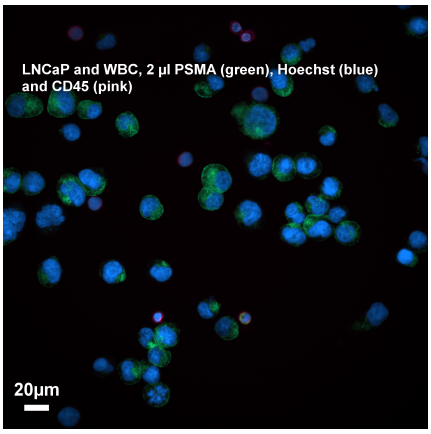
PSMA was titrated on LNCaP cells, with 2 µl of unknown concentration per 100 µl cell suspension chosen as optimal concentration. PSMA was then tested on EpCAM low cell line PC3 along with EpCAM high cell lines MCF-7 and LNCaP. To investigate whether non-specific binding to white blood cells occurred, cell lines were spiked in lysed blood. Results are depicted in figure 6, showing that LNCaP has a high PSMA expression, where PC3 and MCF-7 have a much lower expression. PSMA did not stain white blood cells. Spiked tumour cells (CTC) and white blood cells (WBC) are distinguishable as plotted in the right column of figure 6, with on the x-axis PerCP-labelled CD45 and on the y-axis FITC-labelled PSMA.



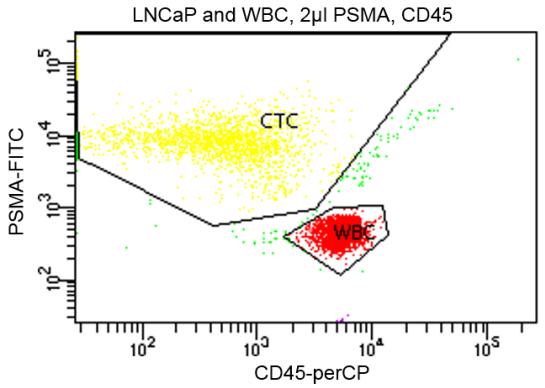
(A) PC3 spiked in blood



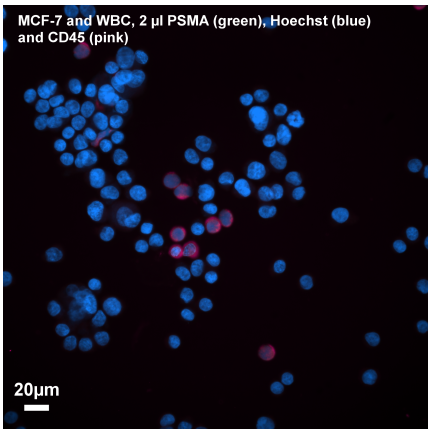
(B) Mean response of PC3: 1,909



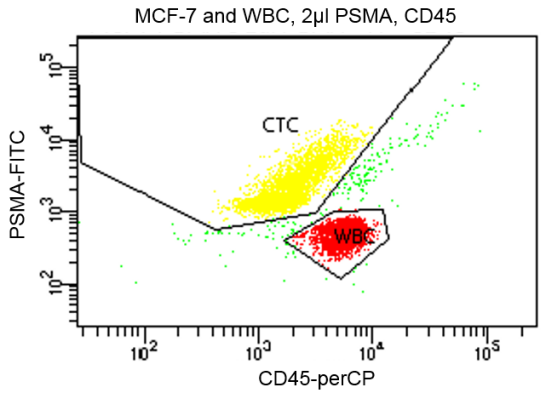
(C) LNCaP spiked in blood



(D) Mean response of LNCaP: 16,175



(E) MCF-7 spiked in blood



(F) Mean response of MCF-7: 2,718

Figure 6: PSMA staining of cancer cells spiked in blood showing that cancer cells and white blood cells can be discriminated based on positive PSMA (FITC/green) and negative CD45 (PerCP/pink) staining, with LNCaP the highest PSMA expressing cell line. Fluorescent microscopic (left) and flow cytometric (right) results from cancer cells spiked in blood, stained with 2 µl anti-PSMA per 100 µl cell suspension, 4 µg/ml Hoechst 33342 and CD45. Fluorescent microscope: anti-PSMA in green, LUT 692-2849, exposure 0.75 s, Hoechst 33342 in blue and CD45 in pink. Flow cytometer scatter plots with PSMA on y-axis, CD45 on x-axis, spiked cancer cells (CTC) in yellow, WBC in red. A and B: Spiked PC3 cells. C and D: Spiked LNCaP cells. E and F: Spiked MCF-7 cells.

3.1.2 E-cadherin

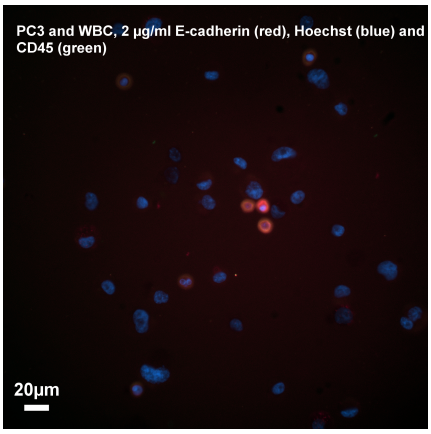
E-cadherin was titrated on LNCaP cells with 2 µg/ml chosen as optimal concentration. E-cadherin was then tested on EpCAM low cell line PC3 along with EpCAM high cell lines MCF-7 and LNCaP. To investigate whether non-specific binding to white blood cells occurred, cell lines were spiked in lysed blood. Results are depicted in figure 7, showing that MCF-7 has the highest expression, LNCaP has high expression and PC3 has a much lower expression of E-cadherin. E-cadherin did not stain white blood cells. Spiked tumour cells (CTC) and white blood cells (WBC) are distinguishable as plotted in the right column of figure 7, with on the x-axis PerCP-labelled CD45 and on the y-axis APC-labelled E-cadherin.

3.1.3 Cadherin-11

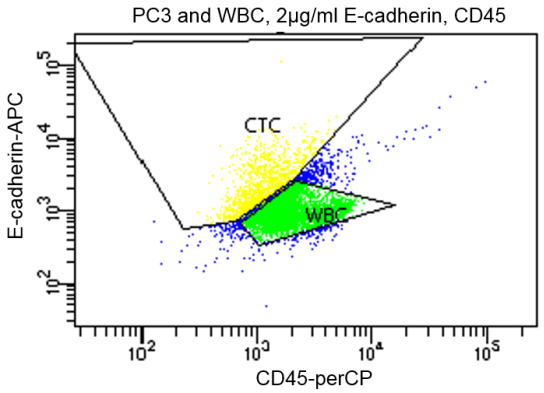
Cadherin-11 expression was tested on the genetically modified PC3 cell lines, where PCα3N cat 5.2 has a very low cadherin-11 expression and PC3αN cat 92.1 has a high cadherin-11 expression. Tests for the optimal staining concentration were performed with two clones of cadherin-11, 16.a.c.15 from Nijmegen and 16G5 from BioLegend, and PE-labelled secondary antibody in solution. Initially, titration did not show a difference between the positive and negative cell line. The secondary antibody was then tested on MCF-7 cells stained with mouse anti-EpCAM for its functioning and it was concluded that the secondary antibody worked. Both genetically modified PC3 cell lines were then grown on slides and tested with the two clones of cadherin-11 and the PE-labelled secondary antibody. With cadherin-11 clone 16G5, signal could barely be separated from background. With cadherin-11 clone 16.a.c.5, a small difference between positive and negative cells was visible. It was then thought that trypsinisation might cause the breakdown of cadherin-11. Trypsin is a proteolytic enzyme which breaks down proteins that enable cell adhesion to the culture flask and to other cells. [83] For staining in solution, cells were then dissociated with EDTA and compared to cells dissociated with trypsin. As shown in figure 8 cells dissociated with EDTA gave higher mean response units compared to cells that were dissociated with trypsin. It is also shown that PC3αN cat 92.1 cells have a higher cadherin-11 expression than PCα3N cat 5.2 cells and cadherin-11 does not stain white blood cells.

3.1.4 N-cadherin

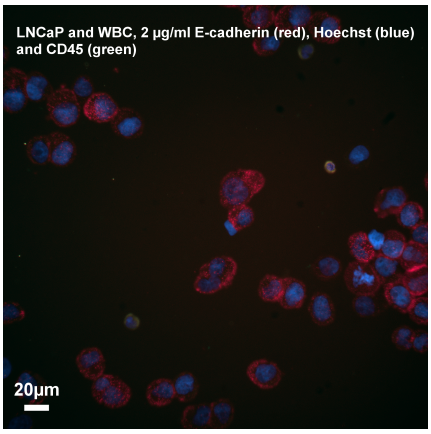
N-cadherin was titrated on PC3 cells. However, no difference between signal and background, positive and negative controls, was obtained. Further experiments with N-cadherin in solution were then performed on living HeLa and HUVEC cells, with MCF-7 cells as negative control. Two different clones for N-cadherin were used, being 8c11 and GC-4. Since no signal was obtained with either one of the clones, a titration for N-cadherin was performed using concentrations ranging from 1 to 50 µg/ml. Staining for the titration experiment was performed on ice, since it was hypothesised that cells internalise the N-cadherin once temperature is raised and their metabolism is activated. [84] Still, no signal was obtained and also not when using tyramide signal amplification on these samples. Cells might be affected by trypsin which was used to dissociate cells from their culture flask, resulting in cleavage of cell surface proteins such as N-cadherin. [83] HeLa and MCF-7 cells that were dissociated with trypsin were then compared to HeLa and MCF-7 cells that were dissociated using 15 mM EDTA. Cells were grown on slides



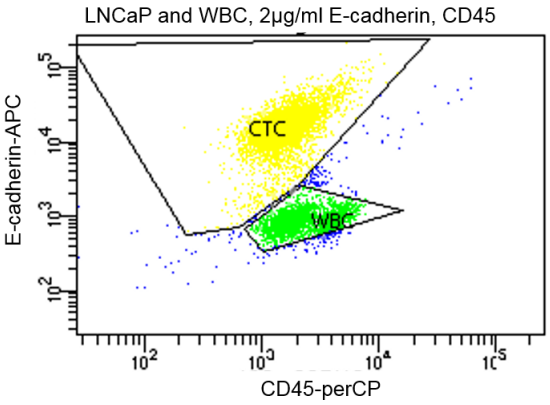
(A) PC3 spiked in blood



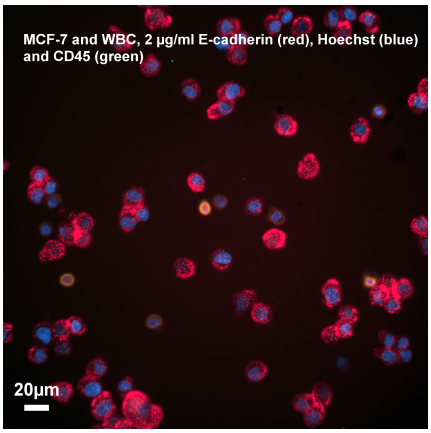
(B) Mean response of PC3: 2,919



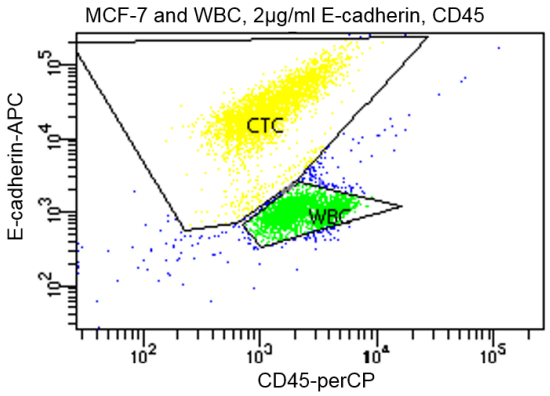
(C) LNCaP spiked in blood



(D) Mean response of LNCaP: 17,914

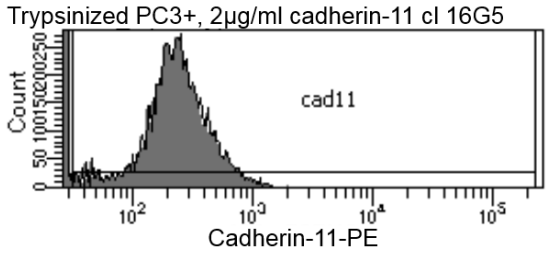


(E) MCF-7 spiked in blood

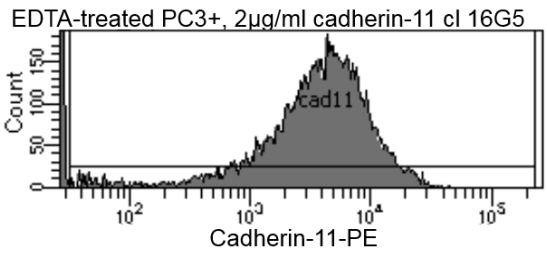


(F) Mean response of MCF-7: 26,303

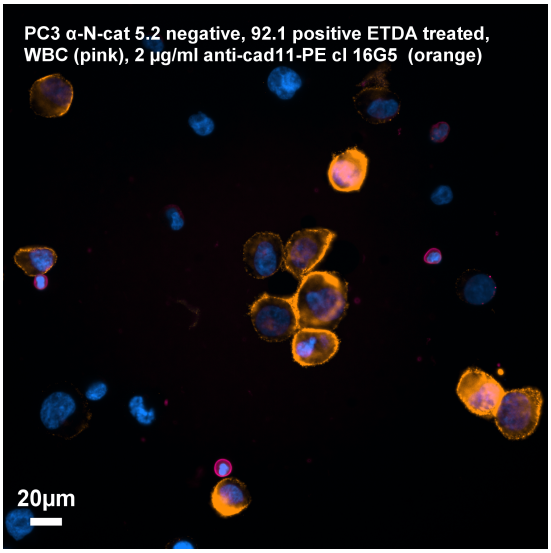
Figure 7: E-cadherin staining of cancer cells spiked in blood showing that cancer cells and white blood cells can be discriminated based on positive E-cadherin (APC/red) and negative CD45 (PerCP/green) staining except for PC3 cells. Highest E-cadherin expression is observed in MCF-7 cells, followed by LNCaP cells. Fluorescent microscopic (left) and flow cytometric (right) results from cancer cells spiked in blood, stained with 2 µg/ml anti-E-cadherin, 4 µg/ml Hoechst 33342 and CD45. Fluorescent microscope: anti-E-cadherin in red, LUT 692-2209, exposure 0.75 s, Hoechst 33342 in blue and CD45 in green. Flow cytometer scatter plots with E-cadherin on y-axis, CD45 on x-axis, spiked cancer cells (CTC) in yellow, WBC in green. A and B: Spiked PC3 cells. C and D: Spiked LNCaP cells. E and F: Spiked MCF-7 cells.



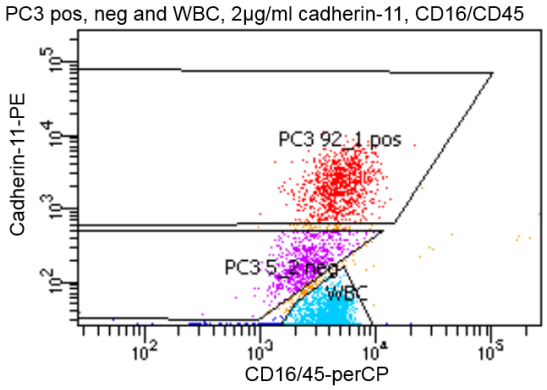
(A) Mean response of PC3+: 288



(B) Mean response of PC3+: 5,325

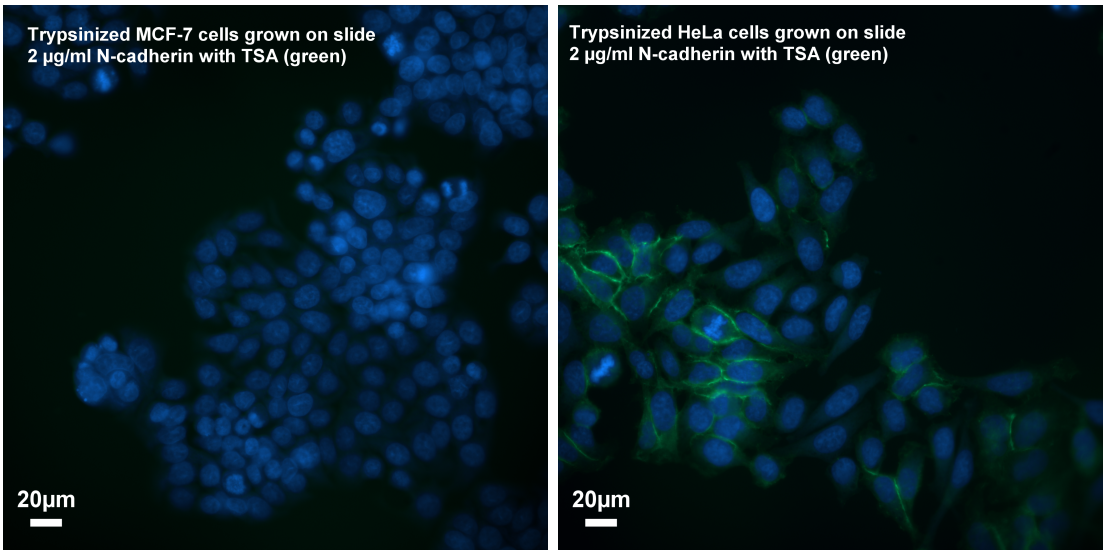


(C) PC3+ and PC3- spiked in blood



(D) Mean response of PC3+: 3,464, PC3-: 174, WBC: 39

Figure 8: Cadherin-11 staining of cancer cells in solution showing that low cadherin-11 expression (PE/orange) is observed in PC3αN cat 5.2 - cells, high expression in PC3αN cat 92.1 + cells when dissociated without trypsin cadherin-11 expression and that cadherin-11 expression is not seen in white blood cells. A: Flow cytometric result of PC3αN cat 92.1 + cells dissociated with trypsin, stained with 2 µg/ml anti-cadherin-11 clone 16G5, goat-anti-mouse IgG-PE and 4 µg/ml Hoechst 33342. B: Flow cytometric result of PC3αN cat 92.1 + cells dissociated with EDTA, stained with 2 µg/ml anti-cadherin-11 clone 16G5, goat-anti-mouse IgG-PE and 4 µg/ml Hoechst 33342. C: Fluorescent microscope image of PC3αN cat 5.2 - and PC3αN cat 92.1 + cells dissociated with EDTA spiked in blood. Anti-cadherin-11 staining with goat-anti-mouse IgG-PE in orange, LUT 294-2489, exposure 0.5 s, Hoechst 33342 in blue and CD16/CD45-PerCP in pink. D: Flow cytometer scatter plot with cadherin-11-PE on y-axis, CD16/45-PerCp on x-axis, PC3αN cat 92.1 + cells in red, PC3αN cat 5.2 - in purple and WBC in blue.



(A) MCF-7 cells grown on cover slip (B) HeLa cells grown on cover slip

Figure 9: N-cadherin staining with TSA on cancer cells grown on cover slips showing that N-cadherin expression (FITC/green) is observed in cell-cell contact areas of HeLa cells and that single cancer cells cannot be detected based on N-cadherin expression. Fluorescent microscopic results from 2 µg/mL N-cadherin staining, anti-mouse HRP incubation and 15 minutes tyramide labelling in green, LUT 1060-16030, exposure 0.75 s, DAPI in blue. A: MCF-7 cells. B: HeLa cells.

and fixed and compared to fixed cells in solution. Cells were stained with 2 µg/ml primary antibody N-cadherin and afterwards TSA was performed. Results are depicted in figure 9. No difference between trypsinised and EDTA treated HeLa cells was seen, neither in solution nor on slides. No differences between positive and negative controls stained in solution were observed by fluorescent microscopy or flow cytometry (figure 15 on page 49). In MCF-7 and HeLa cells grown on slides however, differences were observed. HeLa cells that were in contact with other cells showed N-cadherin expression at the place of cell-cell contact (figure 9 B), whilst this was not observed in MCF-7 cells (figure 9 A).

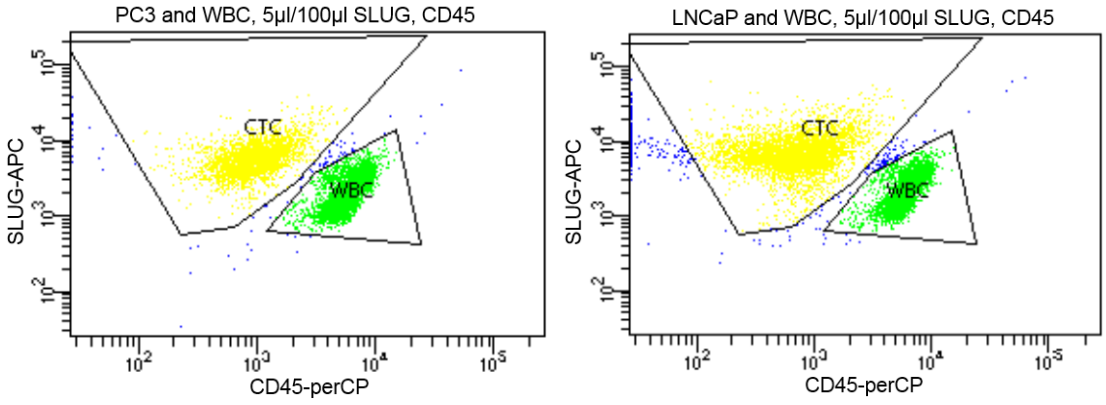
3.1.5 SLUG

SLUG was titrated on PC3 cells, with 5 µl per 100 µl cell suspension chosen as optimal concentration. Using fluorescent microscopy however SLUG was only visible using an exposure time of 3 s and LUT value of 237-432. SLUG was then tested on EpCAM high cell lines MCF-7 and LNCaP along with EpCAM low cell line PC3 and WBC. Spiked tumour cells (CTC) and white blood cells (WBC) could not be discriminated based on anti-SLUG staining as plotted in figure 10, with on the x-axis PerCP-labelled CD45 and on the y-axis APC-labelled SLUG. Under the fluorescent microscope, cancer cells could not be differentiated from white blood cells based on the fluorescent detection of SLUG.

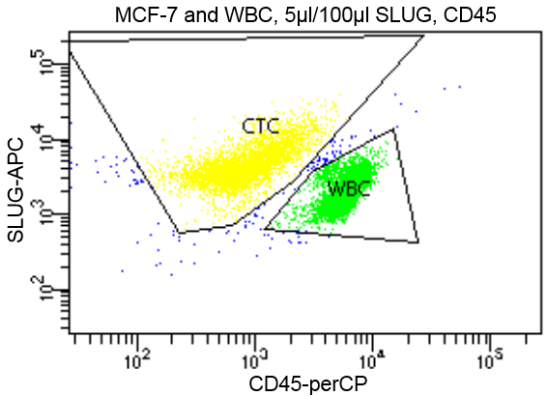
3.1.6 EGFR

EGFR antibody was tested on PC3, MCF-7 and LNCaP cells in different experiments. In none of the experiments, signal could be discriminated from background. Cancer cells

could not be differentiated from white blood cells based on EGFR expression. No further experiments were then performed.



(A) Mean response of PC3: 6,189 (B) Mean response of LNCaP: 7,711



(C) Mean response of MCF-7: 5,326

Figure 10: SLUG staining of cancer cells spiked in blood showing that cancer cells cannot be discriminated from white blood cells based on positive SLUG (APC) and negative CD45 (PerCP) staining. Flow cytometric results from cancer cells spiked in blood stained with 5 µl anti-SLUG per 100 µl cell suspension and CD45. SLUG on y-axis, CD45 on x-axis, spiked cancer cells (CTC) in yellow, WBC in green. A: Spiked PC3 cells. B: Spiked LNCaP cells. C: Spiked MCF-7 cells.

3.2 Signal amplification

Another aim of the project was to enable detection of low abundance markers. For this purpose tyramide signal amplification and proximity ligation assay were investigated.

3.2.1 Tyramide signal amplification

To determine the best parameters for TSA several experiments were performed. Optimisation was performed using the Alexa Fluor 488 Tyramide SuperBoost Kit from Invitrogen. Afterwards, anti-mouse HRP from Invitrogen was compared with anti-FITC:HRP from BioRad, keeping other parameters the same. Experiments were performed *in duplo*. Experiments that were performed and their results are described below.

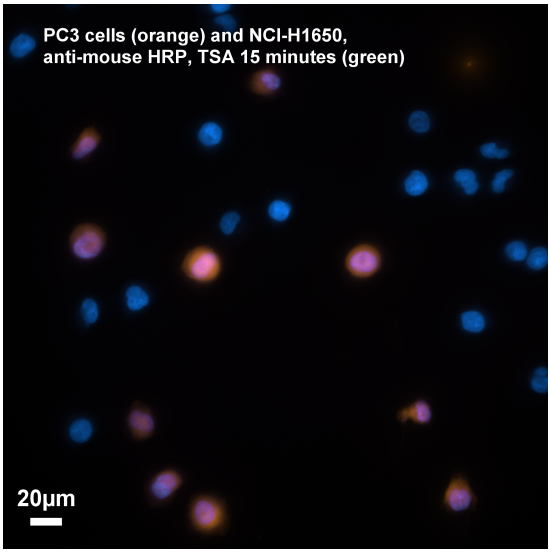
PC3 cells lowly express EpCAM [25]. When cells were stained with 2 µg/ml anti-EpCAM, signal could not be discriminated from background. Therefore, this cell line was used to optimise the tyramide signal amplification protocol. In experiments, primary antibody-independent tyramide signal amplification was observed, which might be caused by endogenous peroxidase activity. To quench endogenous peroxidase activity, a 60 minutes incubation step with 3% H_2O_2 was then incorporated in the protocol according to the user guide of Invitrogen [85].

Three different dilutions of primary EpCAM antibody were investigated, being 2 µg/ml, 0.4 µg/ml and 0.04 µg/ml. With 0.04 µg/ml, signal was the same as background. Using 0.4 µg/ml, a difference between signal and background was observed, but best results were obtained using 2 µg/ml anti-EpCAM.

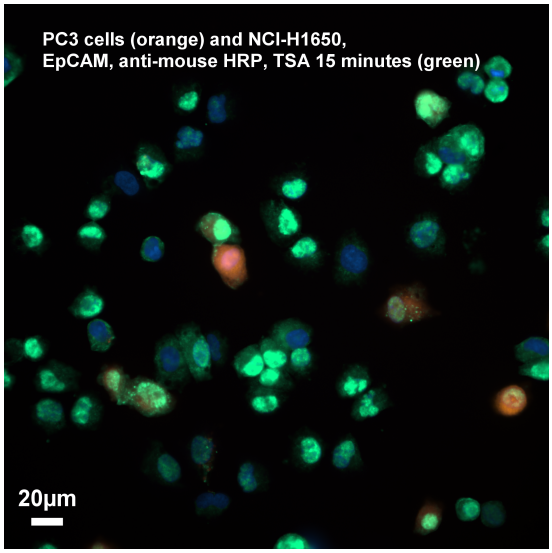
Since low signal was obtained, the incubation step with the tyramide reagent working solution was optimised. PC3 cells and NCI-H1650 cells with an even lower EpCAM expression were then grown on slides together. Incubation times of 5, 10, 15 and 30 min were compared. 15 min tyramide labelling was found to be optimal based on fluorescent microscopic results and therefore incorporated in the protocol.

Then anti-mouse HRP was compared to different dilutions of anti-FITC:HRP. Results are depicted in figure 11. Using 20 µg/ml of anti-FITC:HRP high signal was detected in positive controls as well as in negative controls. The same, though lower, was seen when using 5 µg/ml anti-FITC:HRP. With 1 µg/ml anti-FITC:HRP fluorescent signal was barely detectable in the positive control. 2 µg/ml anti-FITC:HRP was taken as optimal concentration, with visible amplification in positive controls, but not in negative controls (figure 11 c and d). 2 µg/ml anti-FITC:HRP gave results similar to anti-mouse HRP (figure 11 a and b).

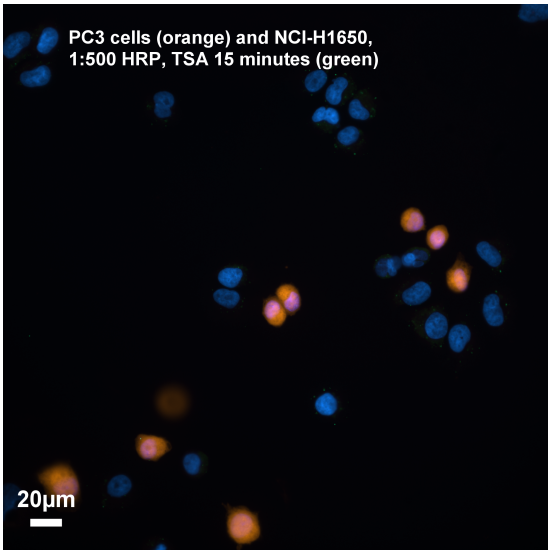
Since circulating tumour cells are in the blood, tyramide signal amplification was tested for its efficacy in solution. On average eight times as high signal was observed in samples with tyramide signal amplification compared to samples with only primary antibody. On slides, the same increase was seen. TSA was also performed on cancer cells spiked in blood. In this experiment a lot of cells were lost during centrifugation and aspiration steps. On white blood cells, that were still present, no TSA signal was observed. EpCAM high cell line MCF-7 was tested along EpCAM low cell line NCI-H1650 grown on slides. Amplification of signal was visible on both cell lines. A clear difference in signal was observed with more than five times as intense signal at MCF-7 cells compared to NCI-H1650. Exact increase could not be calculated since the pixels with MCF-7 signal were saturated.



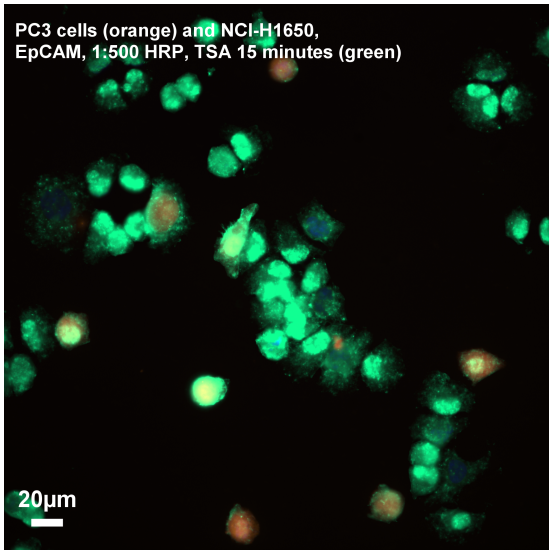
(A) Anti-mouse HRP no primary control



(B) Anti-mouse HRP with anti-EpCAM



(C) 2 µg/mL anti-FITC:HRP no primary control

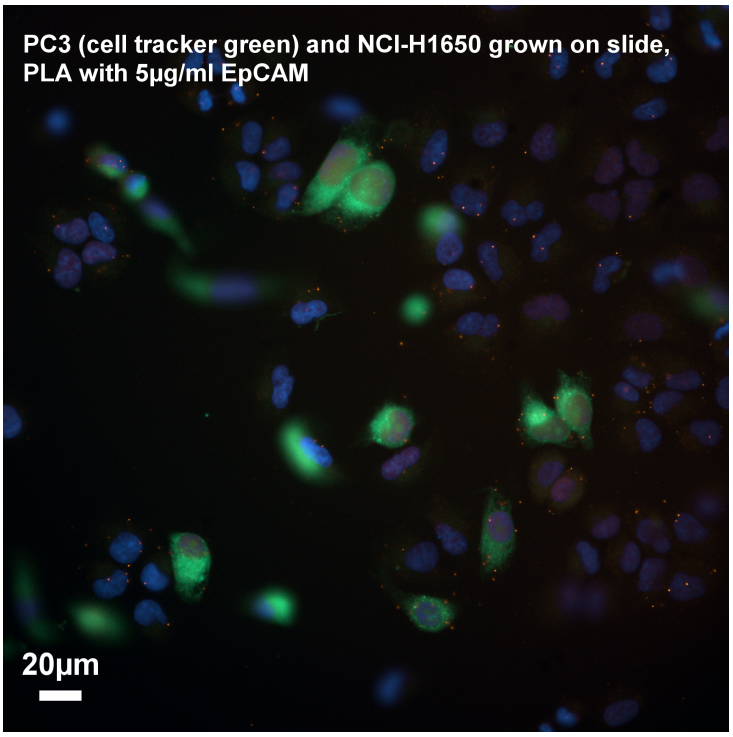


(D) 2 µg/mL anti-FITC:HRP with anti-EpCAM

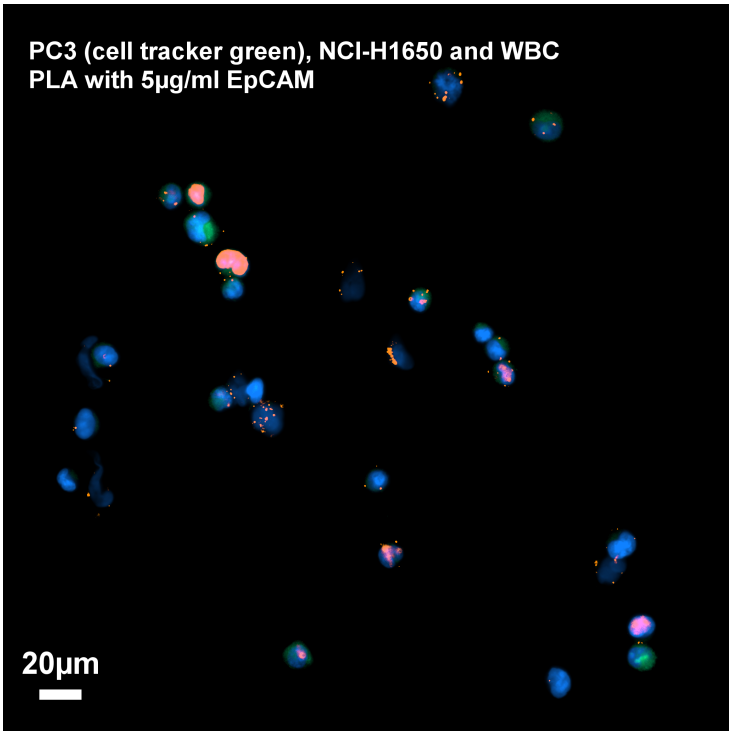
Figure 11: Comparison anti-mouse HRP and anti-FITC:HRP. TSA procedure was optimised with anti-mouse HRP and compared to different concentrations of anti-FITC:HRP. With anti-mouse HRP and with 2 µg/ml anti-FITC:HRP similar results were obtained, keeping other parameters the same. In controls where TSA was performed without primary antibody (A and C) very low signal was observed compared to positive controls (B and D) with primary antibody and TSA. Fluorescent microscopic results from PC3 and NCI-H1650 cells grown on cover slips. Negative controls without primary antibody (left column), positive controls stained with 2 µg/ml anti-EpCAM (right column), anti-mouse HRP (top row) and 2 µg/ml anti-FITC:HRP (bottom row), DAPI (blue, all samples). 15 minutes Alexa Fluor 488 tyramide labelling, LUT 1057-16113, exposure 0.75 s in green. DAPI in blue.

3.2.2 Proximity ligation assay

Protocol optimisation for proximity ligation assay was already performed at MCBP (University of Twente, the Netherlands). According to that protocol, cells were pressed against a positively charged microscopic slide using cytospin at 300xg for 10 minutes. [86] Since a lot of cells were lost with that procedure, cells were then grown on cover slips instead of using cytospin. EpCAM low cell line PC3 and EpCAM very low cell line NCI-H1650 were grown on slides together. On both cell lines, small dots were visible after the proximity ligation assay was completed, see also figure 12. In negative controls without primary antibody no dots were observed. To examine the applicability of proximity ligation assay in solution PC3 cells and NCI-H1650 cells were spiked in blood, while using white blood cells as negative control. Microscopic analysis of 75 cells of each cell type showed a slightly higher number of dots at PC3 cells (on average 10-15 dots) compared to NCI-H1650 cells (on average 5-10 dots) and white blood cells (<5 dots). During the washing steps a lot of cells were lost however.



(A) PLA on cover slip



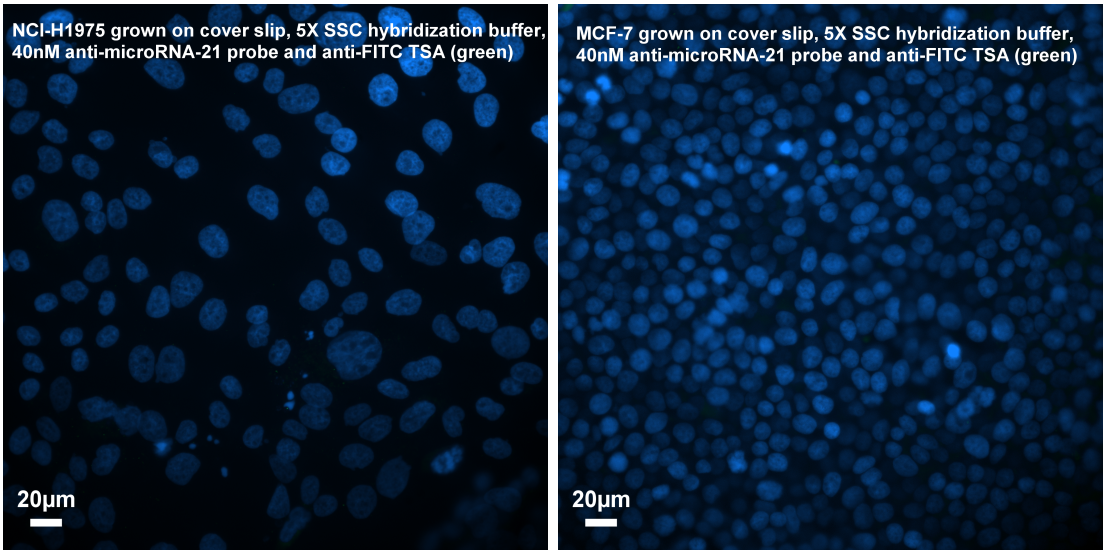
(B) PLA in solution

Figure 12: Fluorescent microscopic results from PLA. PLA was performed to amplify signal of EpCAM staining in solution and on slide on EpCAM low cell line PC3 and EpCAM very low cell line NCI-H1650. On average 10-15 dots in PC3 cells, 5-10 dots in NCI-H1650 cells and <5 dots in white blood cells were counted. Cells incubated overnight with 5 µg/ml anti-EpCAM before performing PLA. PLA dots in orange, PE LUT 589-1414, exposure 0.3 s. DAPI in blue. A: PC3 and NCI-H1650 cells grown on cover slip. B: PC3 and NCI-H1650 cells spiked in blood.

3.3 MicroRNA-21 detection

Using the method of Gasch *et al.* [82] for microRNA-21 detection no difference between microRNA-21 high cell lines MCF-7 and NCI-H1975 and microRNA-21 low cell line NCI-H1650 was observed. Even with TSA, no signal different from background was observed. Stringency washing steps were then adapted to the stringency washing steps as given in section 2.7 and different hybridisation buffers were tested. Despite protocol adaptations microRNA-21 expression was still not observed with FISH and TSA on positive cell lines MCF-7 and NCI-H1975 (see figure 13), with cell line NCI-H1650 as negative control.

The hybridisation ability of the anti-microRNA-21 probe was then tested on PVDF membranes as described in section 2.7. Since microRNA-21 is strongly conserved throughout evolution [87], it was thought that probe hybridised with the fish sperm of the hybridisation mix, reducing the probe available to target the human microRNA-21. Fish sperm was then replaced with BSA. Still no signal was observed and experiments were discontinued. No images were taken.



(A) NCI-H1975 cells grown on cover slip (B) MCF-7 cells grown on cover slip

Figure 13: Anti-microRNA-21 hybridisation with anti-FITC TSA on cancer cells grown on cover slips showing that microRNA-21 (FITC/green) signal is not observed in positive cell lines. Fluorescent microscopic results from 40 nM anti-microRNA-21 hybridisation, anti-FITC HRP incubation and 15 minutes Alexa Fluor 488 tyramide labelling in green, LUT 586-13241, exposure 0.75 s, DAPI in blue. A: NCI-H1975 cells. B: MCF-7 cells.

4 Discussion

The goal of this research project was to determine the presence of EMT related markers on CTC, to improve detection of low abundance markers by using TSA or PLA and to use TSA to detect microRNA in CTC. Parts will be discussed in this order.

Epithelial-to-mesenchymal transition markers

Expression of epithelial antigen EpCAM was compared to expression of epithelial antigen E-cadherin and mesenchymal antigens cadherin-11 and N-cadherin and PSMA in PC3, MCF-7 and LNCaP cells. Expression of epithelial antigen EpCAM is low in the highly metastatic PC3 cell line, whereas EpCAM expression is high in the LNCaP and MCF-7 cell line which have low metastatic potential. [88, 89]

Similar results were obtained for staining with the epithelial marker E-cadherin. E-cadherin expression was high in EpCAM high cell lines MCF-7 and LNCaP and low in EpCAM low cell line PC3. No signal was observed on white blood cells. E-cadherin is an epithelial marker that is downregulated during EMT. [31] It is likely that CTC that lost their EpCAM expression during EMT also lost their E-cadherin expression. Therefore, EpCAM low or negative CTC that are missed in CellSearch might still be missed when using E-cadherin next to EpCAM and cytokeratin, making E-cadherin a less suitable marker.

PSMA can be present in the epithelial, mesenchymal or hybrid state of CTC of prostatic origin as shown in figure 2. EpCAM low prostate cancer cell line PC3 is missed since it does not express PSMA. Other prostate cancer cell lines next to LNCaP and PC3 need to be tested for their PSMA expression. If in most prostate cancer cell lines and prostate cancer patient samples PSMA is expressed it might still be a useful marker next to EpCAM, since prostate cancer is a common disease with more than 1.11 million men estimated to be diagnosed worldwide in 2012 [90].

Cadherin-11 expression was observed using PC3αN cat 92.1 + cells dissociated without trypsin. No non-specific staining with the EMT associated marker cadherin-11 on white blood cells was seen. Other cadherin-11 high and low cell lines can be tested to find out whether there is a correlation between cadherin-11 and EpCAM expression. If cadherin-11 expression is found to be upregulated in EpCAM low or negative cancer cells, it is a suitable marker to use next to EpCAM and cytokeratins to identify tumour cell subpopulations.

For N-cadherin, early experiments were performed on PC3 before continuing experimenting on higher expressing cell line HeLa since no signal was obtained. Multiple parameters were looked at. In none of the experiments with cells in solution signal was obtained that could be discriminated from background, also not when using TSA. In HeLa cells grown on cover slips however, signal using TSA was observed at areas where cells were in contact with each other. In cells that were not in contact with one or more cells no signal was observed. In literature it was found that a small fraction of N-cadherin molecules is concentrated at sites of cell-cell contact, but the majority of N-cadherin molecules are expressed diffusively on the cell membrane. [91–93] Increased signal was found at areas with cell-cell attachment in this project. The diffuse N-cadherin molecules however could not be observed. It might be that the antibody only binds to N-cadherin molecules that interact with N-cadherins of neighbouring cells. This influences the usability for the identification of circulating tumour cells. Many single CTC, but few aggregates of CTC can

be found in metastatic cancer patients [13, 94] making N-cadherin a less suitable marker.

For EGFR and SLUG, signal from cancer cells could not be discriminated from background and white blood cells. Then experiments were discontinued. One might look at another clone and/or brand of the primary antibody. To differentiate non-specific signal from specific antibody signal and determine the level of background in an experiment, it is recommended to use the same concentration of isotype controls along positive controls while experimenting. For experiments with SLUG, it might be possible that anti-SLUG primary antibody could not reach the nucleus where SLUG resides due to unsuccessful permeabilisation. One could investigate a higher concentration of saponin or a stronger permeabilising agent like Triton-X. One should keep in mind however, that permeabilisation damages the membrane, so membrane proteins of interest might be removed as well. Whether SLUG and/or EGFR are good markers to be used next to EpCAM and cytokeratin to identify CTC cannot be concluded based on obtained results and needs to be investigated.

Besides immunostaining, western blot can be used to detect specific proteins in a sample. In general western blots are more sensitive than immunocytochemistry, but no information about the spatial distribution of a protein is obtained. Semi-quantitative results can be obtained when running an internal quantity standard along the samples in the gel. It also enables comparison of protein content between different samples. Just as it is the case with immunostaining, experimental conditions need optimisation.

Recommendations

For future experiments with staining of adhesion molecules such as cadherin, it is recommended to dissociate cells without trypsin since it can result in cleavage of cell surface proteins of interest.

One can perform cadherin-11 and PSMA staining on multiple EpCAM high and low cell lines to see whether there is a correlation between the expression of EpCAM and cadherin-11 and EpCAM and PSMA. For further experiments with EGFR and SLUG antibodies from another clone and/or brand need to be tested. It is likely that with mesenchymal markers such as cadherin-11 more CTC are identified than with only EpCAM and cytokeratins using CellSearch. This was not tested in this project. Whether more CTC are identified can be tested using multiple cadherin-11 high and low expressing cells while also investigating their EpCAM expression. If a correlation is seen between low EpCAM expression and high mesenchymal marker cadherin-11 expression in multiple cell lines, one might continue with testing the filtered CellSearch waste of metastatic cancer patient samples. If cells in the waste can be identified with this additional marker, it increases the total number of CTC found by CellSearch, making cadherin-11 a suitable marker to use along EpCAM and cytokeratins. Whether these EpCAM low or negative cells, that are identified with additional markers, are clinically relevant needs to be investigated. In several cancers EpCAM expressing CTC predict poor outcome. [17,95] A large study needs to be performed on EpCAM low or negative cells found in CellSearch waste in relation to patient outcome. If there is a correlation between patient outcome and EpCAM low or negative cells, these cells can be a useful biomarker for patient prognosis in addition to EpCAM high CTC.

Signal amplification

For protocol optimisation of TSA, it was preferred to have cells on microscopic slides or cover slips. A number of washing steps had to be performed and performing them in solution elongated the procedure significantly due to the centrifugation steps with also a higher chance of cell loss. Growing cells on cover slips took a longer preparation time, but had minimal cell loss as advantage. It took two nights before most cells were attached to the cover slip.

Circulating tumour cells cannot be grown on slides however, making staining in solution or the use of cytospin necessary. With centrifugation at 500xg for 5 minutes and gentle aspiration, cell loss is prevented. Cytospin is a fast procedure to have cells on a slide, however almost all cells were lost during washing. One can look at surface coating of microscopic slides to enhance attachment of cells to the slide during the procedure with cytospin. For detection of low abundance antigens on CTC that are on a microsieve (Vycap, Enschede, The Netherlands, not tested in this project), washing and staining solutions are put on top of the filter and are removed by gently pressing down the sieve on the sponge. Cancer cells cannot pass the filter and will stay on top. It is expected that TSA for cells on microsieves will take as long as TSA for cells grown on cover slips.

It is also interesting to see whether with EpCAM high and low cells more difference in intensity can be observed compared to experiments with EpCAM low and very low cells. Jaros observed differences between EpCAM high and low cell lines. [86] In another experiment with PLA where cancer cells were spiked in blood, in half of the examined white blood cells a few PLA dots were observed. This number might be too high, since white blood cells were not stained with CD45 but assigned by eye to be a white blood cell based on size and morphology, whilst it might be a tumour cell. Jaros [86] concluded that fluorescently labelled probes not only hybridise specifically with the amplified DNA, but also non-specifically bind to white blood cells. When using PLA to quantify low abundance proteins, this might be a problem, since based on these dots cancer cells cannot be discriminated from white blood cells. On cancer cells without primary antibody no dots were observed however, see also figure 14 on page 48. Staining white blood cells with anti-CD45 or CD16 after the PLA procedure is finished, permits discrimination between cancer cells and white blood cells. Counting of dots was done manually, by scrolling through the sample with different focuses of the microscope. This is time consuming and may be imprecise, since a dot can accidentally be missed by human error. No analysis of experiments with PLA was performed at the flow cytometer. According to Leuchowius *et al.* this should be possible. [96] Whether so few dots can be detected can be investigated. Performing flow cytometric analysis using cell lines with different EpCAM expression for PLA, it can be checked whether it is actually possible and also information can be obtained about the detection limit.

With PLA no 100% detection efficiency is obtained. [97, 98] Not all oligos that are in close proximity will give rise to a circular DNA ligation product. [97] This might be due to the probe design, oligo quality or inefficient enzymatic reactions. For the formation of circular DNA in PLA four different oligonucleotides need to assemble and hybridise correctly, see also figure 4 on page 8. This increases the chance that non-circular ligation products are formed. For detection circular products are required. Grannas found that PLA formed both circular and linear ligation DNA products [97]. With UnFold PLA detection efficiency is increased. In UnFold PLA the circularization oligonucleotide is incorporated in the sequence of one of the PLA probes to limit the formation of non-circular ligation products. [97] Since TSA only depends on the affinity of primary and

secondary antibodies and on one enzymatic reaction, TSA might be more efficient.

TSA is based on an enzymatic reaction of HRP, making it time dependent. To obtain reproducible results, control of timing is essential. Although tyramide labelling was timed, it was not totally accurate due to human error. With an increasing number of samples, timing was more imprecise. For PLA, precise timing is less important as long as enough amplification cycles can be completed to obtain signal, see also figure 4. Therefore, samples are better comparable and experiments more reproducible using PLA compared to TSA. PLA also has a better spatial resolution, enabling quantification of single molecules, compared to TSA where more diffuse signal is observed. In literature, it is said that in the vicinity of the HRP molecule, covalent coupling of highly reactive, short-lived tyramide radicals to proteins occurs. This should result in a minimal diffusion-related loss of signal localisation. [60] Compared to PLA, the obtained spatial resolution with TSA was very poor. However, detection efficiency is not known.

Recommendations

It is advisable to use TSA for qualitative measurements only, since precise timing for quantitative measurements is difficult. When one is interested in the spatial distribution of a protein, it is recommended to use PLA rather than TSA, since obtained spatial resolution with TSA is poor. Other PLA designs such as UnFold PLA can be investigated to enhance detection efficiency. Coating to enhance cell attachment on cover slips or microscopic slides can be investigated. Also the applicability of PLA and TSA on CTC captured on microsieves can be studied.

MicroRNA-21 detection

For microRNA-21 detection, a probe was purchased at Exiqon. In the instruction manual of the optimisation kit for miRCURY LNA microRNA ISH, one can find that the kit for optimisation is supplied with a formamide-free hybridisation buffer specifically for miRCURY LNA detection probe-based *in situ* hybridisation. [99] Nevertheless, it is not said of what components their buffer is made. The hybridisation temperature is influenced by the hybridisation buffer used. Using the by Exiqon recommended hybridisation temperature of 56 °C no signal was observed. Hybridisation temperature was adapted to 50 °C according to Gasch *et al.* [82] Still no signal was observed. Even when spotting amplicons of the microRNA-21 gene containing the target sequence on a PVDF membrane, no signal was observed. Either the probe is malfunctioning, or the procedure is suboptimal.

Besides the hybridisation buffer, the Exiqon optimisation kit contains a scramble-microRNA probe which is a negative control probe and a U6 small nuclear RNA probe which is a positive control probe. A positive result from the positive control indicates that the procedure is optimised and working, even if the other cell samples in the same experiment are negative. In that way, positive controls prove negative results to be valid. U6 small nuclear RNA is a non-coding small nuclear RNA component located in the nucleus of eukaryotes.

Recommendations

It is advisable to use U6 small nuclear RNA probe to optimise the protocol parameters such as hybridisation temperature. To determine the optimal hybridisation temperature

with the Exiqon hybridisation buffer, experiments with hybridisation temperatures ranging from 50 to 60 °C can be performed. With different dilutions of the U6 small nuclear RNA probe ranging from 0.1 to 2.0 nM the sensitivity level of the *in situ* hybridisation protocol can be identified. Then detection probe concentration can be optimised with an optimisation range from 20 to 80 nM.

5 Conclusion

To enable identification of EpCAM low or negative circulating tumour cells, different markers were investigated on the cell lines PC3, LNCaP and MCF-7. A high PSMA expression was observed in LNCaP cells and a lower expression in PC3 and MCF-7 cells. Its suitability as marker next to EpCAM in CellSearch needs to be investigated. The highest expression of epithelial marker E-cadherin was observed in MCF-7 cells, followed by LNCaP cells, whereas low expression was observed in PC3 cells. E-cadherin is likely to be downregulated in cells that underwent EMT, reducing its usability as marker next to EpCAM. Signal of EGFR and SLUG was not seen to be different from background. If those markers can be used next to EpCAM needs to be investigated using other antibodies. N-cadherin was only observed in HeLa cells at areas of cell-cell contact, making it a less useful marker. Cadherin-11 was tested on PC3αN cat 5.2 (cadherin-11 low) and PC3αN cat 92.1 (cadherin-11 high) and could be observed when using PC3αN cat 92.1 cells that were dissociated without trypsin. If cadherin-11 is also upregulated in other EpCAM low or negative cell lines, it can be used as marker next to EpCAM in the CellSearch system.

To enhance detection of low abundance proteins, two signal amplification methods were investigated. The first was tyramide signal amplification. Using endogenous peroxidase quenching, 2 µg/ml anti-EpCAM, anti-mouse HRP or 2 µg/ml anti-FITC:HRP and 15 minutes tyramide labelling time, tyramide signal amplification was proven effective for cells in solution and cells grown on slides. Spatial resolution was poor however. The second amplification method was proximity ligation assay, which showed good spatial resolution and enabled single molecule quantification.

To differentiate between normal cells and circulating tumour cells in filtered blood of the CellSearch system, detection of microRNA-21 with fluorescence *in situ* hybridisation in combination with tyramide signal amplification was investigated. No signal was observed, also not when hybridising the probe with amplified microRNA-21 gene material.

References

[1] R. Rubin and D. S. Strayer, *RUBIN'S PATHOLOGY: Clinicopathologic Foundations of Medicine*, 6th ed. Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins, 2012.

[2] R. A. Harouaka, M. Nisic, and S.-Y. Zheng, "Circulating tumor cell enrichment based on physical properties." *Journal of laboratory automation*, vol. 18, no. 6, pp. 455–68, Dec. 2013. [Online]. Available: http://journals.sagepub.com/doi/full/10.1177/2211068213494391

[3] G. P. Gupta and J. Massagué, "Cancer metastasis: building a framework." *Cell*, vol. 127, no. 4, pp. 679–95, Nov. 2006. [Online]. Available: http://www.sciencedirect.com/science/article/pii/S0092867406014140

[4] "WHO — World Health Organization," *WHO*, 2017. [Online]. Available: http://www.who.int/en/

[5] Janssen Diagnostics, "CELLSEARCH® — Home," 2017. [Online]. Available: https://www.cellsearchctc.com/

[6] J. F. Swennenhuis, G. van Dalum, L. L. Zeune, and L. W. M. M. Terstappen, "Improving the CellSearch® system." *Expert review of molecular diagnostics*, vol. 16, no. 12, pp. 1291–1305, Dec. 2016. [Online]. Available: http://www.tandfonline.com/doi/full/10.1080/14737159.2016.1255144

[7] M. Cristofanilli, G. T. Budd, M. J. Ellis, A. Stopeck, J. Matera, R. Ph, M. C. Miller, J. M. Reuben, G. V. Doyle, W. J. Allard, L. W. M. M. Terstappen, and D. F. Hayes, "Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer," *n engl j med*, vol. 351, no. 8, pp. 781–91, 2004. [Online]. Available: http://www.nejm.org/doi/pdf/10.1056/NEJMoa040766

[8] S. C. P. Williams, "Circulating tumor cells." *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 13, p. 4861, Mar. 2013. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/23533270http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3612640

[9] S. Meng, D. Tripathy, E. P. Frenkel, S. Shete, E. Z. Naftalis, J. F. Huth, P. D. Beitsch, M. Leitch, S. Hoover, D. Euhus, B. Haley, L. Morrison, T. P. Fleming, D. Herlyn, L. W. M. M. Terstappen, T. Fehm, T. F. Tucker, N. Lane, J. Wang, and J. W. Uhr, "Circulating Tumor Cells in Patients with Breast Cancer Dormancy," *Clinical Cancer Research*, vol. 10, pp. 8152–8162, 2004. [Online]. Available: http://www.oncoveterinaria.com.ar/contenidos/archivos/file/Julio/Dormancy.pdf

[10] V. Kumar and S. L. S. L. Robbins, *Robbins basic pathology*, 8th ed. Saunders/Elsevier, 2007.

[11] C. Alix-Panabières, S. Mader, K. Pantel, E. Epithelial, and A. Antibodies, "Epithelial-mesenchymal plasticity in circulating tumor cells," *J Mol Med*, vol. 95, pp. 133–142, 2017.

[12] E. E. van der Toom, J. E. Verdone, and K. J. Pienta, "Disseminated tumor cells and dormancy in prostate cancer metastasis," *Current Opinion in Biotechnology*, vol. 40, pp. 9–15, Aug. 2016. [Online]. Available: http://linkinghub.elsevier.com/retrieve/pii/S0958166916300258

[13] "Circulating Tumor Cells," in *Encyclopedia of Cancer*, M. Schwab, Ed. Berlin, Heidelberg: Springer Berlin Heidelberg, 2011, pp. 865–868. [Online]. Available: http://link.springer.com/10.1007/978-3-642-16483-5_1182

[14] M. A. Nieto, R. Y.-J. Huang, R. A. Jackson, and J. P. Thiery, "EMT: 2016," *Cell*, vol. 166, no. 1, pp. 21–45, Jun. 2016. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/27368099http://linkinghub.elsevier.com/retrieve/pii/S0092867416307966

[15] M. Cristofanilli, D. F. Hayes, G. T. Budd, M. J. Ellis, A. Stopeck, J. M. Reuben, G. V. Doyle, J. Matera, W. J. Allard, M. C. Miller, H. A. Fritsche, G. N. Hortobagyi, and L. W. M. M. Terstappen, "Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer." *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, vol. 23, no. 7, pp. 1420–30, Mar. 2005. [Online]. Available: http://ascopubs.org/doi/10.1200/JCO.2005.08.140http://www.ncbi.nlm.nih.gov/pubmed/15735118

[16] J. S. de Bono, H. I. Scher, R. B. Montgomery, C. Parker, M. C. Miller, H. Tissing, G. V. Doyle, L. W. Terstappen, K. J. Pienta, and D. Raghavan, "Circulating Tumor Cells Predict Survival Benefit from Treatment in Metastatic Castration-Resistant Prostate Cancer," *Clinical Cancer Research*, vol. 14, no. 19, 2008. [Online]. Available: http://clincancerres.aacrjournals.org/content/14/19/6302.long

[17] S. De Wit, G. Van Dalum, A. T. M. Lenferink, A. G. J. Tibbe, T. Jeroen, N. Hiltermann, H. J. M. Groen, C. J. M. Van Rijn, and L. W. M. M. Terstappen, "The detection of EpCAM + and EpCAM – circulating tumor cells," *Nature Publishing Group*, 2015. [Online]. Available: https://www.utwente.nl/en/tnw/ctctrap/publications/articles/sreppublication.pdf

[18] M. C. Miller, G. V. Doyle, and L. W. M. M. Terstappen, "Significance of Circulating Tumor Cells Detected by the CellSearch System in Patients with Metastatic Breast Colorectal and Prostate Cancer," *Journal of Oncology*, vol. 8, 2010. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2793426/pdf/JO2010-617421.pdf

[19] W. J. Allard, J. Matera, M. C. Miller, M. Repollet, M. C. Connelly, C. Rao, A. G. J. Tibbe, J. W. Uhr, and L. W. M. M. Terstappen, "Tumor Cells Circulate in the Peripheral Blood of All Major Carcinomas but not in Healthy Subjects or Patients With Nonmalignant Diseases," *Clinical Cancer Research*, vol. 10, no. 20, 2004. [Online]. Available: http://clincancerres.aacrjournals.org/content/10/20/6897\#T2

[20] L. Dean, *Blood groups and red cell antigens*. National Center for Biotechnology Information (US), 2005. [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK2263/

[21] T. M. Gorges, I. Tinhofer, M. Drosch, L. Röse, T. M. Zollner, T. Krahn, and O. von Ahsen, "Circulating tumour cells escape from EpCAM-based detection due

to epithelial-to-mesenchymal transition." *BMC cancer*, vol. 12, no. 1, p. 178, May 2012. [Online]. Available: http://bmccancer.biomedcentral.com/articles/10.1186/1471-2407-12-178

[22] S. D. Mikolajczyk, L. S. Millar, P. Tsinberg, S. M. Coutts, M. Zomorrodi, T. Pham, F. Z. Bischoff, and T. J. Pircher, "Detection of EpCAM-Negative and Cytokeratin-Negative Circulating Tumor Cells in Peripheral Blood." *Journal of oncology*, vol. 2011, p. 252361, Jan. 2011. [Online]. Available: http://europepmc.org/articles/PMC3090615/?report=abstract

[23] O. Gires and N. H. Stoecklein, "Dynamic EpCAM expression on circulating and disseminating tumor cells: causes and consequences." *Cellular and molecular life sciences : CMLS*, vol. 71, no. 22, pp. 4393–402, Nov. 2014. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/25103341

[24] D. T. Miyamoto, L. V. Sequist, and R. J. Lee, "Circulating tumour cells—monitoring treatment response in prostate cancer," *Nature Reviews Clinical Oncology*, vol. 11, no. 7, pp. 401–412, May 2014. [Online]. Available: http://www.nature.com/doifinder/10.1038/nrclinonc.2014.82

[25] "The Human Protein Atlas." [Online]. Available: http://www.proteinatlas.org/

[26] S. V. Litvinov, M. P. Velders, H. A. Bakker, G. J. Fleuren, and S. O. Warnaar, "Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule." *The Journal of cell biology*, vol. 125, no. 2, pp. 437–46, Apr. 1994. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/8163559

[27] E. R. Sherwood, L. A. Berg, N. J. Mitchell, J. E. McNeal, J. M. Kozlowski, and C. Lee, "Differential cytokeratin expression in normal, hyperplastic and malignant epithelial cells from human prostate." *The Journal of urology*, vol. 143, no. 1, pp. 167–71, Jan. 1990. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/1688457

[28] S. A. Joosse, J. Hannemann, J. Spötter, A. Bauche, A. Andreas, V. Müller, and K. Pantel, "Changes in keratin expression during metastatic progression of breast cancer: impact on the detection of circulating tumor cells." *Clinical cancer research : an official journal of the American Association for Cancer Research*, vol. 18, no. 4, pp. 993–1003, Feb. 2012. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/22228641

[29] N. H. Akhtar, O. Pail, A. Saran, L. Tyrell, and S. T. Tagawa, "Prostate-specific membrane antigen-based therapeutics." *Advances in urology*, vol. 2012, p. 973820, 2012. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/21811498http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3145341

[30] L. A. Caromile, K. Dortche, M. M. Rahman, C. L. Grant, C. Stoddard, F. A. Ferrer, and L. H. Shapiro, "PSMA redirects cell survival signaling from the MAPK to the PI3K-AKT pathways to promote the progression of prostate cancer," *Science Signaling*, vol. 10, no. 470, 2017. [Online]. Available: http://stke.sciencemag.org/content/10/470/eaag3326

[31] R. Singhai, V. W. Patil, S. R. Jaiswal, S. D. Patil, M. B. Tayade, and A. V. Patil, "E-Cadherin as a diagnostic biomarker in breast cancer." *North American journal of medical sciences*, vol. 3, no. 5, pp. 227–33, May 2011. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/22558599http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3337742

[32] K. Chu, C.-J. Cheng, X. Ye, Y.-C. Lee, A. J. Zurita, D.-T. Chen, L.-Y. Yu-Lee, S. Zhang, E. T. Yeh, M. C.-T. Hu, C. J. Logothetis, and S.-H. Lin, "Cadherin-11 Promotes the Metastasis of Prostate Cancer Cells to Bone," *Molecular Cancer Research*, vol. 6, no. 8, 2008. [Online]. Available: http://mcr.aacrjournals.org/content/6/8/1259

[33] M. J. Pishvaian, C. M. Feltes, P. Thompson, M. J. Bussemakers, J. A. Schalken, and S. W. Byers, "Cadherin-11 Is Expressed in Invasive Breast Cancer Cell Lines," *Cancer Res.*, vol. 59, no. 4, pp. 947–952, Feb. 1999. [Online]. Available: http://cancerres.aacrjournals.org/content/59/4/947.long

[34] S. Assefnia, S. Dakshanamurthy, J. M. Guidry Auvil, C. Hampel, P. Z. Anastasiadis, B. Kallakury, A. Uren, D. W. Foley, M. L. Brown, L. Shapiro, M. Brenner, D. Haigh, and S. W. Byers, "Cadherin-11 in poor prognosis malignancies and rheumatoid arthritis: common target, common therapies." *Oncotarget*, vol. 5, no. 6, pp. 1458–74, Mar. 2014. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/24681547http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4039224

[35] R. L. Satcher, T. Pan, M. A. Bilen, X. Li, Y.-C. Lee, A. Ortiz, A. P. Kowalczyk, L.-Y. Yu-Lee, and S.-H. Lin, "Cadherin-11 endocytosis through binding to clathrin promotes cadherin-11-mediated migration in prostate cancer cells," *Journal of Cell Science*, vol. 128, no. 24, 2015. [Online]. Available: http://jcs.biologists.org/content/128/24/4629

[36] I. Ramis-Conde, M. A. J. Chaplain, A. R. A. Anderson, and D. Drasdo, "Multi-scale modelling of cancer cell intravasation: the role of cadherins in metastasis," *Physical Biology*, vol. 6, no. 1, p. 016008, Mar. 2009. [Online]. Available: http://stacks.iop.org/1478-3975/6/i=1/a=016008?key=crossref.17bd7385c69264bfd8cd626158359f4b

[37] R. Kalluri and R. A. Weinberg, "The basics of epithelial-mesenchymal transition." *The Journal of clinical investigation*, vol. 119, no. 6, pp. 1420–8, Jun. 2009. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/19487818http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2689101

[38] W. Guo, Z. Keckesova, J. L. Donaher, T. Shibue, V. Tischler, F. Reinhardt, S. Itzkovitz, A. Noske, U. Zürrer-Härdi, G. Bell, W. L. Tam, S. A. Mani, A. van Oudenaarden, and R. A. Weinberg, "Slug and Sox9 Cooperatively Determine the Mammary Stem Cell State," *Cell*, vol. 148, no. 5, pp. 1015–1028, Mar. 2012. [Online]. Available: http://linkinghub.elsevier.com/retrieve/pii/S0092867412001651

[39] K. Tsukasa, Q. Ding, M. Yoshimitsu, Y. Miyazaki, S. Matsubara, and S. Takao, "Slug contributes to gemcitabine resistance through epithelial-mesenchymal transition in CD133+ pancreatic cancer cells," *Human Cell*, vol. 28, no. 4, pp. 167–174, Oct. 2015. [Online]. Available: http://link.springer.com/10.1007/s13577-015-0117-3

[40] M. A. Nieto, "THE SNAIL SUPERFAMILY OF ZINC-FINGER TRANSCRIPTION FACTORS," *Nature Reviews Molecular Cell Biology*, vol. 3, no. 3, pp. 155–166, Mar. 2002. [Online]. Available: http://www.nature.com/doifinder/10.1038/nrm757

[41] J. Grzegrzolka, M. Biala, P. Wojtyra, C. Kobierzycki, M. Olbromski, A. Gomulkiewicz, A. Piotrowska, J. Rys, M. Podhorska-Okolow, and P. Dziegiel, "Expression of EMT Markers SLUG and TWIST in Breast Cancer." *Anticancer research*, vol. 35, no. 7, pp. 3961–8, Jul. 2015. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/26124343

[42] C. Holz, F. Niehr, M. Boyko, T. Hristozova, L. Distel, V. Budach, and I. Tinhofer, "Epithelial–mesenchymal-transition induced by EGFR activation interferes with cell migration and response to irradiation and cetuximab in head and neck cancer cells," *Radiotherapy and Oncology*, vol. 101, no. 1, pp. 158–164, Oct. 2011. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/21665310http://linkinghub.elsevier.com/retrieve/pii/S0167814011002350

[43] K. Jennbacken, T. Tesan, W. Wang, H. Gustavsson, J. Damber, and K. Welén, "N-cadherin increases after androgen deprivation and is associated with metastasis in prostate cancer," *Endocrine-related cancer*, vol. 17, no. 2, pp. 469–79, 2010. [Online]. Available: https://www.readbyqxmd.com/read/20233707/n-cadherin-increases-after-androgen-deprivation-and-is-associated-with-metastasis-in-prostate-can

[44] U. H. Frixen, J. Behrens, M. Sachs, G. Eberle, B. Voss, A. Warda, D. Ltehner, and W. Bircluneier, "E-Cadherin-mediated Cell-Cell Adhesion Prevents Invasiveness of Human Carcinoma Cells," *Journal of cell biology*, vol. 113, no. 1, pp. 173–185, 1991.

[45] Y.-g. Jiang, J.-h. Zhao, Y. Luo, D.-l. He, N. Li, X.-h. Cui, and T. Peng, "Expressions of E-cadherin and beta-catenin in LNCaP and ARCaP cell lines and their significance." *Zhonghua nan ke xue = National journal of andrology*, vol. 15, no. 10, pp. 867–71, Oct. 2009. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/20112731

[46] Y.-C. Lee, C.-J. Cheng, M. Huang, M. A. Bilen, X. Ye, N. M. Navone, K. Chu, H.-H. Kao, L.-Y. Yu-Lee, Z. Wang, and S.-H. Lin, "Androgen depletion up-regulates cadherin-11 expression in prostate cancer." *The Journal of pathology*, vol. 221, no. 1, pp. 68–76, May 2010. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/20191612http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2936767

[47] J.-C. Pignon, B. Koopmansch, G. Nolens, L. Delacroix, D. Waltregny, and R. Winkler, "Androgen Receptor Controls EGFR and ERBB2 Gene Expression at Different Levels in Prostate Cancer Cell Lines," *Cancer Research*, vol. 69, no. 7, 2009.

[48] J. Rae, J. Scheys, K. Clark, R. Chadwick, M. Kiefer, and M. Lippman, "EGFR and EGFRvIII expression in primary breast cancer and cell lines," *Breast Cancer Research and Treatment*, vol. 87, pp. 87–95, 2004. [Online]. Available: https://www.researchgate.net/publication/8336768_EGFR_and_EGFRvIII_expression_in_primary_breast_cancer_and_cell_lines

[49] M. Emadi Baygi, Z.-S. Soheili, F. Essmann, A. Deezagi, R. Engers, W. Goering, and W. A. Schulz, "Slug/SNAI2 regulates cell proliferation and invasiveness of metastatic

prostate cancer cell lines." *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 31, no. 4, pp. 297–307, Aug. 2010. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/20506051

[50] Z.-Q. Wu, X.-Y. Li, C. Y. Hu, M. Ford, C. G. Kleer, and S. J. Weiss, "Canonical Wnt signaling regulates Slug activity and links epithelial-mesenchymal transition with epigenetic Breast Cancer 1, Early Onset (BRCA1) repression." *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 41, pp. 16 654–9, Oct. 2012. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/23011797http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3478591

[51] S. Tai, Y. Sun, J. M. Squires, H. Zhang, W. K. Oh, C.-Z. Liang, and J. Huang, "PC3 is a cell line characteristic of prostatic small cell carcinoma," *The Prostate*, vol. 71, no. 15, pp. 1668–1679, Nov. 2011. [Online]. Available: http://doi.wiley.com/10.1002/pros.21383

[52] B. Frigerio, G. Fracasso, E. Luison, S. Cingarlini, M. Mortarino, A. Coliva, E. Seregni, E. Bombardieri, G. Zuccolotto, A. Rosato, M. Colombatti, S. Canevari, and M. Figini, "A single-chain fragment against prostate specific membrane antigen as a tool to build theranostic reagents for prostate cancer," *European Journal of Cancer*, vol. 49, no. 9, pp. 2223–2232, 2013. [Online]. Available: http://www.sciencedirect.com/science/article/pii/S0959804913000877

[53] M. Heine, B. Freund, P. Nielsen, C. Jung, R. Reimer, H. Hohenberg, U. Zangemeister-Wittke, H.-J. Wester, G. H. Lüers, and U. Schumacher, "High Interstitial Fluid Pressure Is Associated with Low Tumour Penetration of Diagnostic Monoclonal Antibodies Applied for Molecular Imaging Purposes," *PLoS ONE*, vol. 7, no. 5, p. e36258, May 2012. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/22590529http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3348149http://dx.plos.org/10.1371/journal.pone.0036258

[54] "LNCaP." [Online]. Available: https://www.dsmz.de/catalogues/details/culture/ACC-256.html

[55] "Phycobiliproteins—Section 6.4." [Online]. Available: https://www.thermofisher.com/nl/en/home/references/molecular-probes-the-handbook/ultrasensitive-detection-technology/phycobiliproteins.html

[56] "Microspheres—Section 6.5." [Online]. Available: https://www.thermofisher.com/nl/en/home/references/molecular-probes-the-handbook/ultrasensitive-detection-technology/microspheres.html

[57] F. Takizawa, J. P. Kinet, and M. Adamczewski, "Binding of phycoerythrin and its conjugates to murine low affinity receptors for immunoglobulin G." *Journal of immunological methods*, vol. 162, no. 2, pp. 269–72, Jun. 1993. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/8315294

[58] ThermoFisher, "Ultrasensitive Detection Technology," in *Molecular Probes Handbook*, 11th ed. Thermo Fisher Scientific, 2011, ch. 6, pp. 191–235. [Online]. Available: https://www.thermofisher.com/nl/en/home/references/molecular-probes-the-handbook/ultrasensitive-detection-technology.html

[59] Molecular Probes, "Tyramide Signal Amplification Kits," 2013.

[60] ThermoFisher Scientific, "TSA and Other Peroxidase-Based Signal Amplification Techniques—Section 6.2," 2017. [Online]. Available: https://www.thermofisher.com/nl/en/home/references/molecular-probes-the-handbook/ultrasensitive-detection-technology/tyramide-signal-amplification-technology.html\#

[61] R. P. Haugland, "Ultrasensitive Detection Technology - TSA," in *Handbook of Flouresecebt Probes abd Research Products*. Molecular Probes, 2002, ch. 6.2, pp. 152–159. [Online]. Available: http://www.mobitec.de/probes/docs/sections/0602.pdf

[62] B. Koos, L. Andersson, C. M. Clausson, K. Grannas, A. Klaesson, G. Cane, and O. Söderberg, "Analysis of protein interactions in situ by proximity ligation assays," *Current Topics in Microbiology and Immunology*, vol. 377, pp. 111–126, 2014. [Online]. Available: http://link.springer.com/10.1007/82_2013_334

[63] S. M. Gustafsdottir, E. Schallmeiner, S. Fredriksson, M. Gullberg, O. Söderberg, M. Jarvius, J. Jarvius, M. Howell, and U. Landegren, "Proximity ligation assays for sensitive and specific protein analyses," *Analytical Biochemistry*, vol. 345, no. 1, pp. 2–9, Oct. 2005. [Online]. Available: http://linkinghub.elsevier.com/retrieve/pii/S000326970500062X

[64] Sigma-Aldrich, "Amplified Detection Duolink ® Proximity Ligation Assay (PLA)," 2017. [Online]. Available: http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/General_Information/1/amplified-detection-duolink-pla.pdf

[65] "User Guide Duolink ® In Situ – Fluorescence." [Online]. Available: http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Instructions/1/duolink-fluorescence-user-manual.pdf

[66] Biotek, "PLA," 2017. [Online]. Available: https://www.biotek.com/assets/tech_resources/13906/figure2.jpg

[67] J. A. Chan, A. M. Krichevsky, and K. S. Kosik, "MicroRNA-21 Is an Antiapoptotic Factor in Human Glioblastoma Cells," *Cancer Research*, vol. 65, no. 14, 2005. [Online]. Available: http://cancerres.aacrjournals.org/content/65/14/6029.long

[68] S. Feng, S. Cong, X. Zhang, X. Bao, W. Wang, H. Li, Z. Wang, G. Wang, J. Xu, B. Du, D. Qu, W. Xiong, M. Yin, X. Ren, F. Wang, J. He, and B. Zhang, "MicroRNA-192 targeting retinoblastoma 1 inhibits cell proliferation and induces cell apoptosis in lung cancer cells," *Nucleic Acids Research*, vol. 39, no. 15, pp. 6669–6678, Aug. 2011. [Online]. Available: https://academic.oup.com/nar/article-lookup/doi/10.1093/nar/gkr232

[69] J. Gagan, B. K. Dey, R. Layer, Z. Yan, and A. Dutta, "MicroRNA-378 targets the myogenic repressor MyoR during myoblast differentiation." *The Journal of biological chemistry*, vol. 286, no. 22, pp. 19 431–8, Jun. 2011. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/21471220http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3103322

[70] S. D. Selcuklu, M. T. Donoghue, and C. Spillane, "miR-21 as a key regulator of oncogenic processes," *Biochemical Society Transactions*, vol. 37, no. 4, 2009. [Online]. Available: http://www.biochemsoctrans.org/content/37/4/918

[71] X. Xu, A. J. Kriegel, X. Jiao, H. Liu, X. Bai, J. Olson, M. Liang, and X. Ding, "miR-21 in ischemia/reperfusion injury: a double-edged sword?" *Physiological Genomics*, vol. 46, no. 21, 2014. [Online]. Available: http://physiolgenomics.physiology.org/content/46/21/789

[72] K. A. Cissell, Y. Rahimi, S. Shrestha, E. A. Hunt, and S. K. Deo, "Bioluminescence-based detection of microRNA, miR21 in breast cancer cells." *Analytical chemistry*, vol. 80, no. 7, pp. 2319–25, Apr. 2008. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/18302417

[73] H. Zhang, P. Li, H. Ju, M. Pesta, V. Kulda, W. Jin, M. Cai, C. Liu, H. Wu, J. Xu, Y. Ye, G. Zhang, E. Xu, J. Cai, M. Lai, D. Xia, J. Yang, and Y. Wu, "Diagnostic and Prognostic Value of microRNA-21 in Colorectal Cancer: An Original Study and Individual Participant Data Meta-analysis," *Cancer Epidemiology and Prevention Biomarkers*, vol. 23, no. 12, 2014. [Online]. Available: http://cebp.aacrjournals.org/content/23/12/2783

[74] S. M. Hammond, S. Boettcher, A. A. Caudy, R. Kobayashi, and G. J. Hannon, "Argonaute2, a link between genetic and biochemical analyses of RNAi." *Science (New York, N.Y.)*, vol. 293, no. 5532, pp. 1146–50, Aug. 2001. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/11498593

[75] Weizmann Institute of Science, "GeneCards - Human Genes — Gene Database — Gene Search." [Online]. Available: http://www.genecards.org/

[76] A. A. Caudy, R. F. Ketting, S. M. Hammond, A. M. Denli, A. M. P. Bathoorn, B. B. J. Tops, J. M. Silva, M. M. Myers, G. J. Hannon, and R. H. A. Plasterk, "A micrococcal nuclease homologue in RNAi effector complexes," *Nature*, vol. 425, no. 6956, pp. 411–414, Sep. 2003. [Online]. Available: http://www.nature.com/doifinder/10.1038/nature01956

[77] G. Cuccato, A. Polynikis, V. Siciliano, M. Graziano, M. di Bernardo, and D. di Bernardo, "Modeling RNA interference in mammalian cells," *BMC Systems Biology*, vol. 5, no. 1, p. 19, Jan. 2011. [Online]. Available: http://bmcsystbiol.biomedcentral.com/articles/10.1186/1752-0509-5-19

[78] J. Martinez and T. Tuschl, "RISC is a 5' phosphomonoester-producing RNA endonuclease." *Genes & development*, vol. 18, no. 9, pp. 975–80, May 2004. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/15105377http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC406288

[79] M. Seike, A. Goto, T. Okano, E. D. Bowman, A. J. Schetter, I. Horikawa, E. A. Mathe, J. Jen, P. Yang, H. Sugimura, A. Gemma, S. Kudoh, C. M. Croce, and C. C. Harris, "MiR-21 is an EGFR-regulated anti-apoptotic factor in lung cancer in never-smokers." *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 29, pp. 12 085–90, Jul. 2009. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/19597153http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2715493

[80] Z. Nikiforova, M. Taipov, I. Kudryavtsev, and V. Shevchenko, "Association of miR21 and miR155 with Regulation of 15HPGD mRNA in Human Breast Cancer Cells," *ISSN Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry Original Russian Text © Z.N. Nikiforova, M.A.Taipov, I.A. Kudryavtsev, V.E. Shevchenko*, vol. 9, no. 2, pp. 159–165, 1990. [Online]. Available: https://link.springer.com/content/pdf/10.1134\%2FS1990750815020110.pdf

[81] MCBP, "Medical Cell BioPhysics," Enschede, 2017.

[82] C. Gasch, P. N. Plummer, L. Jovanovic, L. M. McInnes, D. Wescott, C. M. Saunders, A. Schneeweiss, M. Wallwiener, C. Nelson, K. J. Spring, S. Riethdorf, E. W. Thompson, K. Pantel, and A. S. Mellick, "Heterogeneity of miR-10b expression in circulating tumor cells." *Scientific reports*, vol. 5, p. 15980, Nov. 2015. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/26522916http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4629160http://dx.doi.org/10.1038/srep15980$\backslash$nhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4629160\&tool=pmcentrez\&rendertype=abstract

[83] H.-L. Huang, H.-W. Hsing, T.-C. Lai, Y.-W. Chen, T.-R. Lee, H.-T. Chan, P.-C. Lyu, C.-L. Wu, Y.-C. Lu, S.-T. Lin, C.-W. Lin, C.-H. Lai, H.-T. Chang, H.-C. Chou, and H.-L. Chan, "Trypsin-induced proteome alteration during cell subculture in mammalian cells." *Journal of biomedical science*, vol. 17, no. 1, p. 36, May 2010. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/20459778http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2873939

[84] N. L. Carrodus, K. S.-L. Teng, K. M. Munro, M. J. Kennedy, and J. M. Gunnersen, "Differential labeling of cell-surface and internalized proteins after antibody feeding of live cultured neurons." *Journal of visualized experiments : JoVE*, no. 84, p. e51139, Feb. 2014. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/24561550http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4123487

[85] Invitrogen, "Tyramide SuperBoost Kits with Alexa Fluor Tyramides," 2016.

[86] A. Jaros, "The evaluation of technology for the characterization of tumor cells." University of Twente, MCBP, Enschede, Tech. Rep., 2017.

[87] "Stem-loop sequence hsa-mir-21." [Online]. Available: http://mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0000077

[88] S. M. Pulukuri, C. S. Gondi, S. S. Lakka, A. Jutla, N. Estes, M. Gujrati, and J. S. Rao, "RNA interference-directed knockdown of urokinase plasminogen activator and urokinase plasminogen activator receptor inhibits prostate cancer cell invasion, survival, and tumorigenicity in vivo." *The Journal of biological chemistry*, vol. 280, no. 43, pp. 36 529–40, Oct. 2005. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/16127174http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1351057

[89] E. A. Pérez-Yépez, J.-T. Ayala-Sumuano, A. M. Reveles-Espinoza, and I. Meza, "Selection of a MCF-7 Breast Cancer Cell Subpopulation with High Sensitivity to IL-1 β : Characterization of and Correlation between Morphological and Molecular Changes Leading to Increased Invasiveness," *International Journal*

of Breast Cancer, vol. 2012, pp. 1–12, May 2012. [Online]. Available: http://www.hindawi.com/journals/ijbc/2012/609148/

[90] "Cancer Research UK." [Online]. Available: https://www.cancerresearchuk.org/

[91] N. L. Tran, R. B. Nagle, A. E. Cress, and R. L. Heimark, "N-Cadherin Expression in Human Prostate Carcinoma Cell Lines An Epithelial-Mesenchymal Transformation Mediating Adhesion with Stromal Cells," *The American Journal of Pathology*, vol. 155, pp. 787–798, 1999. [Online]. Available: http://ajp.amjpathol.org/article/S0002-9440(10)65177-2/pdf

[92] F. Monier-Gavelle and J. Duband, "Control of N-cadherin-mediated intercellular adhesion in migrating neural crest cells in vitro," *Journal of Cell Science*, vol. 108, pp. 3839–3853, 1995. [Online]. Available: http://jcs.biologists.org/content/joces/108/12/3839.full.pdf

[93] P. C. Letourneau, T. A. Shattuck, F. K. Roche, M. Takeichi, and V. Lemmon, "Nerve growth cone migration onto Schwann cells involves the calcium-dependent adhesion molecule, N-cadherin," *Developmental Biology*, vol. 138, no. 2, pp. 430–442, Apr. 1990. [Online]. Available: http://linkinghub.elsevier.com/retrieve/pii/0012160690902092

[94] E. H. Cho, M. Wendel, M. Luttgen, C. Yoshioka, D. Marrinucci, D. Lazar, E. Schram, J. Nieva, L. Bazhenova, A. Morgan, A. H. Ko, W. M. Korn, A. Kolatkar, K. Bethel, and P. Kuhn, "Characterization of circulating tumor cell aggregates identified in patients with epithelial tumors." *Physical biology*, vol. 9, no. 1, p. 016001, Feb. 2012. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/22306705http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3387999

[95] H. Schneck, B. Gierke, F. Uppenkamp, B. Behrens, D. Niederacher, N. H. Stoecklein, M. F. Templin, M. Pawlak, T. Fehm, H. Neubauer, and D. C. C. N. D. N. Duesseldorf, "EpCAM-Independent Enrichment of Circulating Tumor Cells in Metastatic Breast Cancer," *PLOS ONE*, vol. 10, no. 12, p. e0144535, Dec. 2015. [Online]. Available: http://dx.plos.org/10.1371/journal.pone.0144535

[96] K.-J. Leuchowius, I. Weibrecht, and O. Söderberg, "In Situ Proximity Ligation Assay for Microscopy and Flow Cytometry," *Current Protocols in Cytometry*, vol. Chapter 9, p. Unit 9.36, Apr. 2011. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/21455970http://doi.wiley.com/10.1002/0471142956.cy0936s56

[97] K. Grannas, "Improvements and Applications of in situ Proximity Ligation Assays," 2015. [Online]. Available: https://uu.diva-portal.org/smash/get/diva2:801280/FULLTEXT01.pdf

[98] S. Fredriksson, M. Gullberg, J. Jarvius, C. Olsson, K. Pietras, S. M. Gústafsdóttir, A. Östman, and U. Landegren, "Protein detection using proximity-dependent DNA ligation assays," *Nature Biotechnology*, vol. 20, no. 5, pp. 473–477, May 2002. [Online]. Available: http://www.nature.com/doifinder/10.1038/nbt0502-473

[99] "miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) Detection 2 miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) · Instruction

Manual," 2016. [Online]. Available: https://www.exiqon.com/ls/Documents/Scientific/miRCURY-LNA-microRNA-ISH-Optimization-Kit-manual.pdf

Appendix A Immunostaining protocol

1. Spin cells down for 5 minutes at 500xg directly after harvesting and aspirate supernatant

2. Resuspend cell pellet in 1% formaldehyde in PBS 1X and incubate for 15 minutes at room temperature.

3. Spin down at 500xg, aspirate supernatant and resuspend in 1% BSA in PBS 1X to a concentration of $1x10^6$ cells/ml.

4. Bring 500.000 cells per sample in an Eppendorf tube.

5. *Optional: permeabilisation of cells* Spin down at 500xg, aspirate supernatant and resuspend in 0.05% saponin with 1% BSA in PBS 1X and incubate for 10 minutes at room temperature

6. Spin down at 500xg, aspirate supernatant and resuspend in (eventually 0.05% saponin) 1% BSA in PBS 1X and staining reagents to a total volume of 100 µl. Incubate for 30 minutes at 37 °C, protected from light when using fluorescent labeled primary antibodies.

7. *Optional: secondary antibody* Spin down at 500xg, aspirate supernatant and resuspend in 2 ml 1% BSA in PBS 1X. Incubate for 5 minutes at room temperature and spin down at 500xg. Aspirate supernatant and resuspend in 1% BSA in PBS 1X with staining reagents to a total volume of 100 µl. Incubate for 30 minutes at 37 °C, protected from light.

8. Spin down at 500xg, aspirate supernatant and resuspend in 100 µl 1% BSA in PBS 1X with Hoechst 33342 to an end concentration of 4 µg/ml and in case of white blood cells also with CD45. Incubate for 15 minutes at 37 °C, protected from light.

9. Perform fluorescent microscopic analysis or flow cytrometric analysis.

Appendix B TSA protocol

For TSA in solution, fixed cells were used.
Preparation for experiments on cover slips:

1. Incubate cover slips in an opened wells plate with 70% ethanol for at least 30 minutes in a sterile environment (laminar flow hood).
2. Wash cover slips twice with PBS 1X.
3. Add correct cell medium and 40.000 cells/cm^2 and let cells attach to the cover slips at 37 °C and 5% CO_2.
4. After attachment of cells, wash them twice with PBS 1X and fix the cells with 1% formaldehyde in PBS 1X for 30 minutes at room temperature.
5. Rinse twice with PBS 1X.

TSA on cells in solution and on cover slips:

6. Quench endogenous peroxidase activity with 3% H_2O_2 for 60 minutes at room temperature and rinse three times with PBS 1X.
7. Incubate 30 minutes at 37 °C with blocking buffer.
8. Stain cells according to the experiment setup with primary antibodies with mouse as host or labelled with FITC for 30 minutes at 37 °C.
9. Rinse three times with PBS 1X for 10 minutes.
10. Incubate samples with anti-mouse or anti-FITC:HRP for 30 minutes at 37 °C.
11. Wash samples three times with PBS 1X and prepare reaction buffer, H_2O_2 solution, reaction stop working solution and tyramide working solution. Prepare fresh on the day of use and prepare tyramide working solution not longer than 2 hours before use.
    - reaction buffer: one drop to 1 ml ultra-pure water
    - H_2O_2 solution: one drop to 1 ml ultra-pure water
    - reaction stop working solution: 1:11 dilution in PBS 1X
    - tyramide working solution: reaction buffer, H_2O_2 solution and tyramide working solution in a ratio of 100:1:1.
12. Incubate samples with tyramide working solution for 15 minutes at room temperature. Control of time is crucial for reproducibility, since enzyme reactions are time dependent.
13. Add reaction stop working solution after 15 minutes and incubate for 5 minutes. Wash samples three times with PBS 1X for 5 minutes.
14. Place a drop of mounting medium with DAPI on a microscope slide and flip the cover slip on top. Add Hoechst to the samples in solution. Incubate for 15 minutes at room temperature and perform microscopic analysis.

Appendix C PLA protocol

For PLA in solution, fixed cells were used.
Preparation for experiments on cover slips:

1. Incubate cover slips in an opened wells plate with 70% ethanol for at least 30 minutes in a sterile environment (laminar flow hood).
2. Wash cover slips twice with PBS 1X.
3. Add correct cell medium and 40.000 cells/cm^2 and let cells attach to the cover slips at 37 °C and 5% CO_2.
4. After attachment of cells, wash them twice with PBS 1X and fix the cells with 1% formaldehyde in PBS 1X for 30 minutes at room temperature.
5. Rinse twice with PBS 1X.

PLA on cells in solution and on cover slips (reagents, except EpCAM, from Duolink):

6. Incubate samples for 30 minutes at 37 °C with blocking solution.
7. Tap off blocking solution and incubate samples with primary antibodies diluted to desired concentration with antibody diluent (used: 5 µg/ml EpCAM with mouse as host) overnight at 4 °C.
8. Wash slides twice in wash buffer A 1x for 5 minutes.
9. Incubate samples for 60 minutes at 37 °C with PLA probes PLUS and MINUS, diluted in antibody diluent. For 40 µl reaction, use 24 µl antibody diluent, 8 µl PLA probe PLUS stock (5x) and 8 µl PLA probe MINUS stock (5x).
10. Wash samples twice with wash buffer A 1x for 5 minutes under gentle agitation.
11. For ligation, dilute ligation stock 1:5 and ligase stock 1:40 in ultra- pure water just before use. For 40 µl reaction, use 31 µl ultra-pure water, 8 µl of ligation stock (5x) and 1 µl of ligase stock. Incubate samples for 30 minutes at 37 °C.
12. Wash samples twice with wash buffer A 1x for 2 minutes under gentle agitation.
13. For amplification, dilute amplification stock 1:5 and polymerase stock 1:80 in ultra-pure water just before use. For 40 µl reaction, use 31.5 µl ultra-pure water, 8 µl of amplification stock (5x) and 0.5 µl of polymerase stock. Incubate samples for 100 minutes at 37 °C.
14. Wash samples twice with wash buffer B 1x for 10 minutes. Then, wash samples with wash buffer B 0.01x for 1 minute.
15. Place a drop of mounting medium with DAPI on a microscope slide and flip the cover slip on top. Add Hoechst to the samples in solution. Incubate for 15 minutes at room temperature and perform microscopic analysis.

Appendix D Supplementary figures

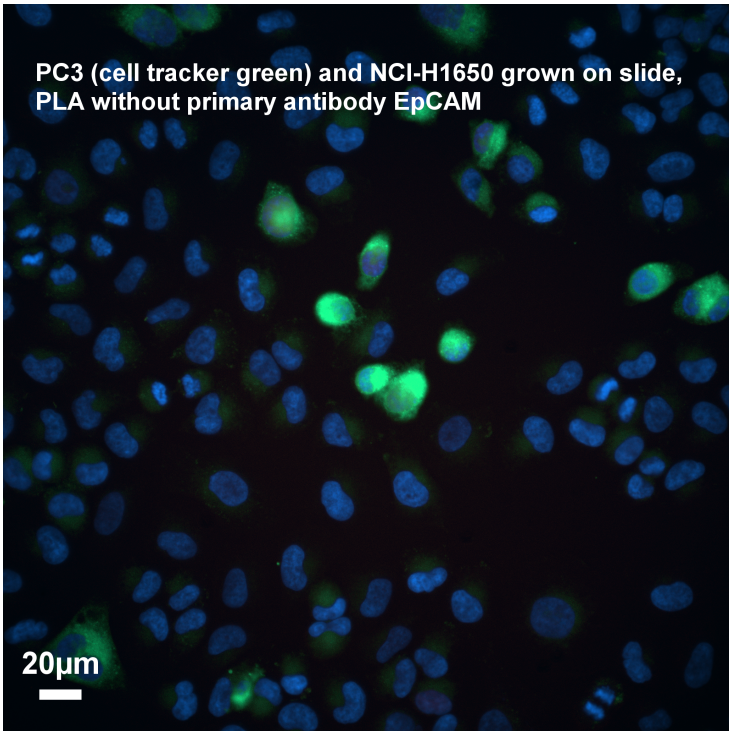
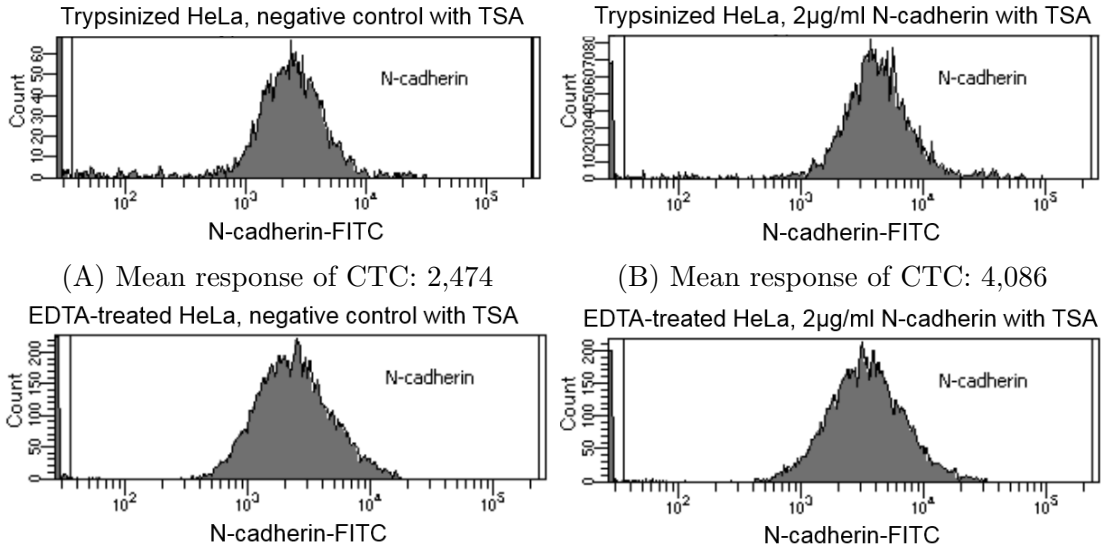


Figure 14: Fluorescent microscopic results from PLA showing that PLA dots cannot be observed when no primary antibody staining is applied. PC3 and NCI-H1650 cells grown on cover slip. PLA procedure was performed without primary antibody as negative control. DAPI in blue. PLA dots in orange, PE LUT 589-1414, exposure 0.3 s.



(A) Mean response of CTC: 2,474

(B) Mean response of CTC: 4,086

(C) Mean response of CTC: 2,013

(D) Mean response of CTC: 3,117

Figure 15: N-cadherin-TSA staining of cancer cells in solution and negative controls showing that mean flow cytometric responses do not differ between positive and negative controls. Flow cytometric results from cancer cells in solution that underwent TSA with or without 2 µg/mL primary N-cadherin staining. Anti-mouse TSA against N-cadherin antibody. A: HeLa cells dissociated with trypsin, negative control of TSA without primary N-cadherin staining. B: HeLa cells dissociated with trypsin, stained with N-cadherin and TSA. C: HeLa cells dissociated without trypsin, negative control of TSA without primary N-cadherin staining. D: HeLa cells dissociated without trypsin, stained with N-cadherin and TSA.

Appendix E TSA optimisation steps

To optimise the TSA procedure multiple parameters were investigated, described here below.

Parameter	**Outcome**
Incubation of cover slips with poly-L-lysine	No difference in cell attachment between treated and non-treated slips
Quenching endogenous peroxidase activity	Without quenching primary antibody independent TSA signal was observed
Primary antibody concentration: 0.04 µg/mL, 0.4 µg/mL or 2 µg/mL	2 µg/mL gave best results
Tyramide labelling time: 5, 10, 15 and 30 minutes	15 minutes was optimal
Anti-FITC:HRP concentration: 1, 2, 5 and 20 µg/ml	2 µg/ml gave results comparable to anti-mouse TSA
TSA in solution versus on cover slips	Both working, though in solution more time consuming