Optimization of immunofluorescence protocols for detection of biomarkers in colorectal and breast cancer tissues

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Optimization of immunofluorescence protocols for detection of biomarkers in formalin-fixed paraffin embedded colorectal and breast cancer tissues

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Cancer remains an undetermined question for modern medicine. Every year millions of people ranging from children to adult die since the modern treatment is unable to meet the challenge. Research must continue in the area of new biomarkers for tumors. Molecular biology has evolved during last years; however, this knowledge has not been applied into the medicine. Biological findings should be used to improve diagnostics and treatment modalities.

In this thesis, human formalin-fixed paraffin embedded colorectal and breast cancer samples were used to optimize the double immunofluorescence staining protocol. Also, immunohistochemistry was performed in order to visualize expression patterns of each biomarker. Concerning double immunofluorescence, feasibility of primary antibodies raised in different and same host species was also tested.

Finally, established methods for simultaneous multicolor immunofluorescence imaging of formalin-fixed paraffin embedded specimens were applied for the detection of pairs of potential biomarkers of colorectal cancer (EGFR, pmTOR, pAKT, Vimentin, Cytokeratin Pan, Ezrin, E-cadherin) and breast cancer (Securin, PTTG1IP, Cleaved caspase 3, ki67).

Keywords: Colorectal cancer, breast cancer, immunohistochemistry, immunofluorescence, biomarker.

ABBREVIATIONS

Ab	antibody
Anti-EGFR mAb	epidermal growth factor receptor monoclonal antibody
BC	breast cancer
BRAF	human gene that makes a protein called B-Raf
CC	cell culture
CRC	colorectal cancer
EGFR	epidermal growth factor receptor
FFPE	formalin-fixed paraffin embedded
HRP	horseradish peroxidase
IF	immunofluorescence
IHC	immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene homolog
LN	lymph node
MAPK	mitogen-activated protein kinase
2-ME	2-mercaptoethanol
Ms	mouse
pAKT	phosphorylated protein kinase B
PI3K	phosphatidylinositide 3-kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
p-mTOR	phosphorylated mammalian target of rapamycin
PTEN	protein tyrosine phosphatase enzyme
Rb	rabbit
SDS	sodium dodecyl sulfate
TMA	tissue microarrays
TSA	tyramide signal amplification

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1. REVIEW OF THE LITERATURE

Cancer, malignant disease originating from epithelial cells, is characterized by aggressive cell growth, invasion and metastasis - an ability to spread to other parts of the body (www.cancer.gov, 2015). Cancer is the major cause of morbidity and mortality in the population: 14 million new diagnoses and 8.2 million cancer deaths were reported worldwide in 2012 (Bernard et al., 2013).

1.1 Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer worldwide (World Cancer Research Fund 2007). Around the world, approximately 1 million of people are diagnosed with colorectal cancer every year, one fourth of them have metastatic disease at the time of diagnosis and another one fourth will develop metastases during the course of their disease (Parkin et al., 2002). Life expectancy of metastasized colorectal carcinoma remains short in spite of new treatment modalities (Wang et al., 2011). Therefore, research on tumor markers to improve the diagnostics, detection of residual disease, and prognostic and predictive features is extensive (Beachy and Repasky, 2008).

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase commonly overexpressed or mutated in many human cancers, including e.g. carcinomas of the breast, lung, head and neck, bladder, kidney, ovary and colorectum. EGFR and its downstream components mediate growth promoting signaling and thus, serve as a target for molecular therapy (Figure 1). (Krasinskas, 2011). EGFR signaling pathway is activated when a ligand binds EGFR and induces dimerization of the receptor, resulting in the activation of tyrosine kinase. The intracellular tyrosine kinase residues participate in an autophosphorylation process that leads to the activation of multiple signal transduction pathways. The two main signaling pathways controlled by EGFR are the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase- (PI3K-) protein kinase B (AKT) pathway. These two signaling pathways lead to activation of several transcription factors that regulate proliferation, differentiation, migration, and apoptosis. (Citri and Yarden, 2006.) Failure in the signaling system promotes cell growth, malignancy, metastatic dissemination and resistance to apoptosis (Krasinskas, 2011).

Treatment of colorectal cancer consists of several possible modalities and, depending on the dissemination of the disease (tumor stage). In metastatic cases, surgical

excision of the tumor can be associated with chemotherapy, radiotherapy, targeted therapy or a combination of different treatments. Targeted therapies include drugs inhibiting VEGF of EGFR signaling. (www.cancer.org, 2015.) Several downstream effectors of EGFR have been shown to have a predictive value for anti-EGFR treatment (Neumann et al, 2013). For example, KRAS, a downstream molecule in EGFR signaling cascade, has been detected with a mutation in 40 % of CRC cases (Bouche et al., 2010). Patients with activating KRAS mutation are resistant to epidermal growth factor receptor monoclonal antibody (anti-EGFR mAb) treatment (Lievre et al., 2006). Additionally, only every third of patients exhibiting wild-type KRAS will benefit from anti-EGFR mAb treatment (Chang et al., 2009). In literature, metastatic cancers with wild-type KRAS and BRAF mutations have been associated with shorter overall survival (Laurent-Puig et al., 2009). Among metastatic colorectal carcinomas that represent/exhibit the KRAS wild type, 5% to 15% can harbor BRAF mutations and, thus, have resistance to anti-EGFR therapy (Loupakis et al., 2009). Additionally, the PI3K-AKT signaling pathway may not function correctly because of mutations in PIK3CA gene, inactivation of protein tyrosine phosphatase enzyme (PTEN) gene or activation of p-AKT (Hynes and Lane, 2005). For example, patients with a mutation in exon 20 of PIK3CA gene do not benefit from targeted treatment using monoclonal antibodies (De Roock et al., 2010). PTEN is a protein that inhibits the function of PI3K (Hynes and Lane, 2005). Therefore, loss of PTEN leads to activation of the PI3K-AKT pathway (Laurent-Puig et al., 2009). p-AKT expression is associated with low stage of the cancer and favourable prognosis (Baba et al., 2011).

Obviously, accurate information on the mutations and their concordance with the expression of EGFR signaling molecules in CRC are needed for improved diagnostics and assessment of patient outcome.

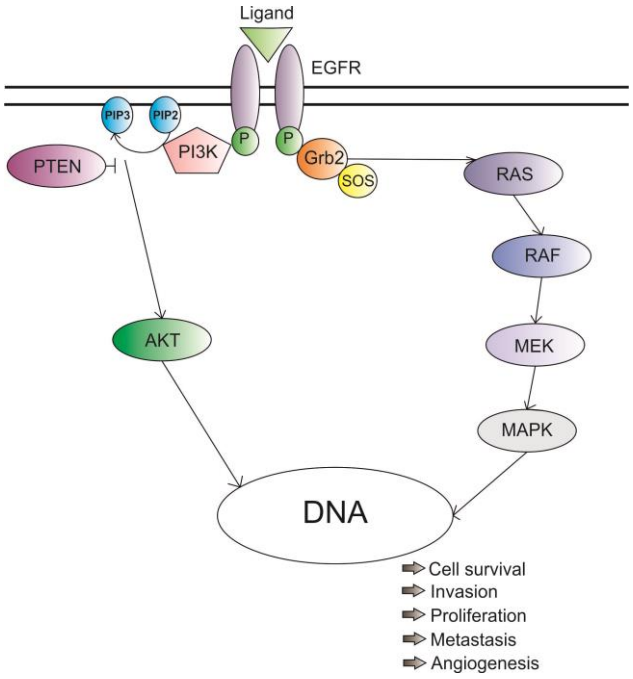


Figure 1. EGFR signaling pathway. Modified from Krasinskas, 2011. EGFR signaling pathway is activated when a ligand binds EGFR and induces dimerization of the receptor, resulting in the activation of tyrosine kinase. The intracellular tyrosine kinase residues participate in an autophosphorylation process that leads to the activation of multiple signal transduction pathways. The two main signaling pathways controlled by EGFR are the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase- (PI3K-) protein kinase B (AKT) pathway. These two signaling pathways lead to activation of several transcription factors that regulate proliferation, differentiation, migration, and apoptosis. (Citri and Yarden, 2006.)

1.2 Breast cancer

Breast cancer (BC) is the most common cancer in women worldwide, and about 1.7 million new cases were diagnosed in 2012 (Ferlay et al., 2012).

Breast cancer is clinically a heterogeneous disease where the outcome of the patient is dependent on several features, particularly the histological type and grade, tumor size, nodal involvement and Estrogen Receptor (ER) and Human Gene of Epidermal Growth Factor Receptor 2 (HER2) status. Many new classification methods have been proposed but up till now in clinical routine the diagnostic and therapeutic decision-making is still based on the traditional histological type and grade with expression status of hormone receptors and HER2 (Lakhani et al., 2012). More recently, gene expression profiling has provided a molecular basis for breast cancer classification (Perou et al., 2000). Currently surrogate immunohistochemical criteria are applied to further allocate invasive breast carcinomas into one of the intrinsic subtypes

corresponding to the molecular classification (Goldhirsch et al., 2011). In routine clinical diagnostics, breast cancer can be classified into Luminal A, Luminal B, HER2-enriched, Claudin-low, Basal-like and Normal Breast-like groups (Figure 2) (Prat and Perou, 2011). The use of the intrinsic classification is grounded by the observed significant survival difference between the patient subgroups (Sorlie et al., 2003). Routine clinical diagnostics based on ER, PR and HER2 is insufficient to predict sensitivity to treatment. Luminal type of breast cancer is thought to be a derivative from normal luminal breast epithelium. Overexpression of ER and low expression of HER2 are distinctive for luminal A type and for this reason it is sensitive to hormonal therapy and associated with long-term survival. (Hu et al., 2006.) Luminal B type, in turn, is characterized by high expression of HER2, low expression of ER, high proliferation and less favourable prognosis (Sorlie et al., 2001).

Traditional breast cancer biomarkers are ER, Progesterone Receptor (PR) and HER2. Hormone receptor-positive carcinomas are best treated with hormonal therapy whereas patients lacking ER and PR-receptors are commonly considered not to benefit from hormonal treatment. According to literature, recurrences in ER-positive carcinomas occur only after 10-20 years and, consequently, ER is an established biomarker for favourable prognosis in breast cancer (Harris et al., 2007.) Approximately 12-15% of breast carcinomas exhibit HER2-amplification. The HER2 protein facilitates cancer cell growth and, as a result, aggressive behavior and progression of breast carcinoma. Patients with HER2-amplified breast carcinomas are offered a special Her2-targeted treatment, most commonly trastuzumab. According to literature, ER- and PR-positive breast carcinomas are less aggressive than HER2-positive carcinomas. The most aggressive breast cancer subtype is the so called triple-negative cancer which is ER/PR-negative and lacks HER2-amplification. This carcinoma subtype shows particularly rapid growth and high risk of recurrence (Foulkes et al., 2010). Triple-negative breast carcinomas are more common in young women (<40 years) and often associated with BRCA1 mutations (Young, 2009). BRCA1 and 2 are tumor suppressor genes, which are normally expressed in breast and several other tissues. In case of BRCA1/2 mutation, the normal DNA repair mechanism called homologous recombination is hampered predisposing the cell to increased mutational load and malignant progression (Friedenson, 2007). Ki67 is a marker of cell proliferation. Ki67 immunohistochemistry has relative prognostic value, especially in luminal B subtype of breast carcinomas. According to several reports, the prognostic value of Ki-67 is, however, limited due to varying inter-laboratory and observer reproducibility (Inwald et al., 2013.)

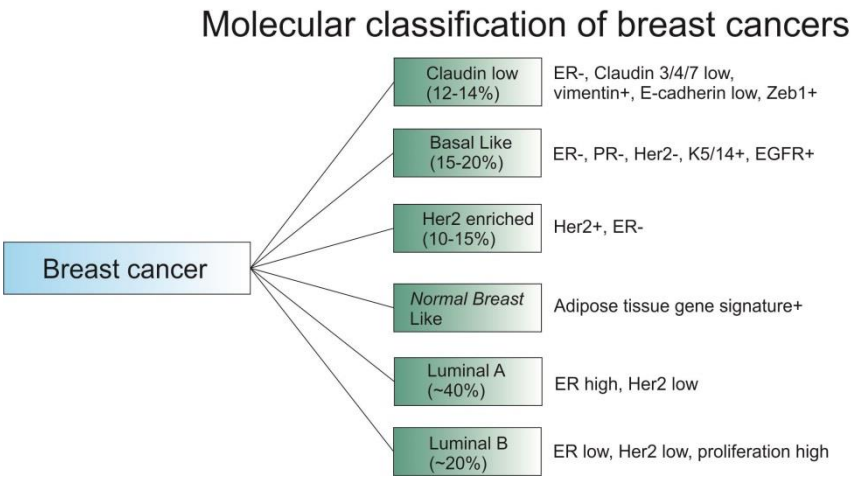


Figure 2. Molecular classification of breast cancer. Modified from Malhotra et al.,2010.

According to most recent gene expression studies, breast cancer classification includes Luminal A, Luminal B, HER2-enriched, Claudin-low, Basal-like and Normal Breast-like groups (Figure 2). (Prat and Perou, 2011.). A subtype of triple-negative breast carcinomas, namely basal carcinoma has attracted attention in the recent years due to its aggressive course of disease. Gene expression pattern of basal like resembles that of basal cells of the normal breast epithelium and expresses cytokeratins 5/6 and 17, integrin 4 and laminin. Basal subtype of breast carcinoma is regularly triple-negative, with frequent mutations in the BRCA1, BRCA2, ATM and TP53 genes, RB1 loss and cyclin E1 amplification (Cancer Genome Atlas Network, 2012.). Low expression of genes involved in tight junctions and cell-cell adhesion, such as claudins and E-cadherin are represented in the recently discovered Claudin-low subgroup of breast carcinomas which is also associated with markers of epithelial-to-mesenchymal transition (EMT) (Herschkowitz et al., 2007.) Both of these breast carcinoma subtypes are associated with unfavourable outcome of disease (Prat et al., 2010). In addition to the above mentioned progress, research in proteomics and gene-expression analysis is constantly facilitating discovery of new biomarkers in breast cancer. Numerous biomarkers are presently under investigation, for example related to the p53, RB, PI3K/Akt/mTOR and Ras/MAPK pathways, as well as alterations associated with genetic instability and epigenetics including histone methylation and acetylation, DNA methylation and microRNAs expression (Kourea et al., 2015). Clearly, further research and new biomarkers are needed for more accurate diagnostics and treatment, as well as for better understanding the biology of this heterogeneous disease.

1.3 Principle of immunohistochemical staining method

Much biological, chemical and molecular information about disease can be obtained from formalin-fixed paraffin embedded (FFPE) tissue samples. Along with conventional morphology, immunohistochemistry (IHC) is a microscope-based technique of wide clinical use. The technique aims to identify specific cellular biomarker molecules, which are not visible using common hematoxylin and eosin staining. (Brandtzaeg, 1998.) In the area of cancer diagnostics, IHC is used to detect tumor markers to assess, whether the tumor is benign or malignant, to evaluate the origin of the tumor, to establish prognosis, or to predict the effect of cancer treatment. (Harsh, 2005.)

Preparation of FFPE specimen consists of tissue fixation, processing and sectioning. In general outline, immunohistochemical staining consists of slide preparation, specific reactions applying primary antibodies and detection of bound antibodies. More precisely, basic procedure usually includes antigen retrieval, different blocking steps, primary antibody incubation in specific solution at appropriate concentration, detection of primary antibody, counterstaining and mounting. (Brandtzaeg, 1998.) In IHC, primary antibody denotes the antibody which specifically recognizes the target protein of interest. The secondary antibody, on the other hand, recognizes the primary antibody. Secondary antibodies are labelled with e.g. enzymes. There are different types of chromogens serving as substrates for these enzymes enabling the visualization of the antigen-antibody complex by light microscopy. (Taylor et al., 2013.)

There are several choices for the detection of primary antibodies. The ability of avidin to bind with high affinity and specificity to biotin is utilized in avidin-biotin complex method. Biotin can bind four avidin molecules. Therefore, the usage of secondary antibody conjugated with biotin, following by the application enzyme-avidin complex amplifies the signal derived from primary antibody. Alkaline phosphatase or horseradish peroxidase (HRP) is typically used as an enzymatic label in this method. Streptavidin-biotin method is based on avidin-biotin method. However, complex is smaller in size and, hence, reaches more easily difficult to access targets. Using phosphatase-anti-phosphatase (PAP) method even higher sensitivity is achieved. In this method, unconjugated secondary antibodies are applied, resulting in attachment of two secondary antibodies to one primary antibody (Figure 3). A tertiary antibodies conjugated with peroxidase is then used to detect secondary antibodies. Multiple tertiary antibodies bind to each secondary antibody amplifying the signal greatly. (Snider, 2014.)

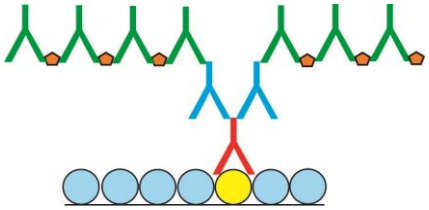


Figure 3. Principle of signal amplification utilizing phosphatase-anti-phosphatase (PAP) method. Target antigen (yellow) is recognized by primary antibody (red), which is recognized by two secondary antibodies (blue). A tertiary antibodies conjugated with peroxidase (green) is then used to detect secondary antibodies, amplifying the signal greatly. Modified from Snider, 2014.

To avoid a well-known limitation of avidin-biotin system, i.e. background staining due to endogenous biotin in tissue, and to further increase signal intensity, modern polymer-based detection techniques have been introduced. These methods apply a polymer backbone conjugated with several secondary antibodies and reporter enzymes (Chilosi et al., 1994). If even higher intensity is required, tyramide signal amplification system (TSA) can be used. It has been reported to increase the signal 50 times higher compared to that detected without amplification. In this technique peroxidase is utilized to activate tyramide which is then covalently bound to proteins in tissue immediately adjacent to the sites where peroxidase is immobilized (Figure 4). Multiple depositions of activated tyramides occur in short period of time amplifying the original signal greatly. Tyramide derivatives are most often fluorescently labeled. However, hapten labelled tyramides and choromogenic substrates have also been introduced. (www.perkinelmer.com.)

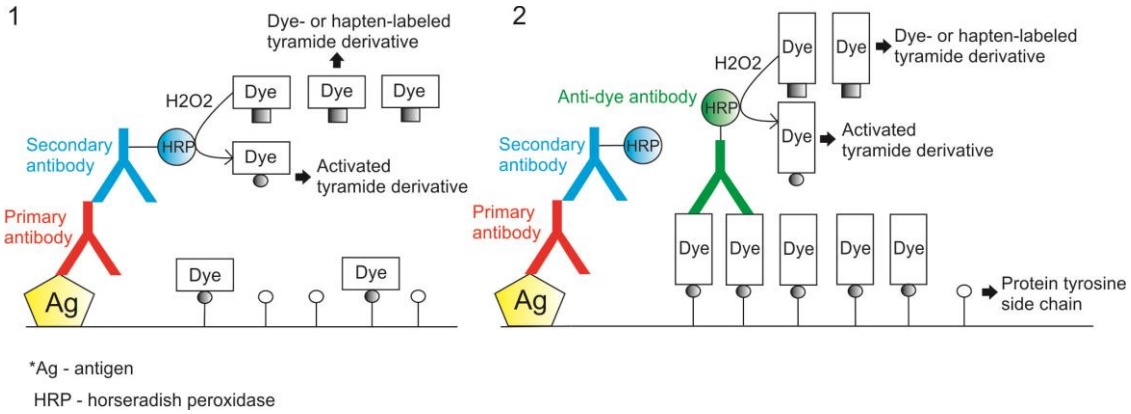


Figure 4. Schematic representation of TSA detection method. The antigen is detected by a primary antibody, followed by a horseradish peroxidase HRP-labeled secondary antibody in connection with a dye-labeled tyramide, resulting in localized deposition of the activated tyramide derivative (Stage 1). Further dye deposition, and consequently higher levels of signal amplification, can be constituted by detecting dye deposited in Stage 1 with a HRP-labeled anti-dye antibody in conjunction with a dye-labeled tyramide (Stage 2). Modified from thermofisher.com.

In IHC, strong staining of background is occasionally seen unrelated to target signal and interfering with the interpretation of the result. For example, staining methods based on biotin, or peroxidase and phosphatase reporters are prone to show high background staining if endogenous forms of these molecules have not been quenched. Other possibilities causing background staining are cross-reactivity and nonspecific binding of an antibody. Nonspecific binding of primary antibody to nontarget epitopes is often caused by high concentration of primary antibody. (Snider, 2014.) Additional blocking step using, for example, bovine serum albumin (BSA) or milk may be needed to avoid this problem (Duhamel and Johnson, 1985.)

Another problem associated sometimes with IHC is a weak target signal, meaning too weak intensity of the signal due to suboptimal conditions used in staining. Weak staining of a target has been associated with enzyme-substrate reactivity, primary antibody potency and secondary antibody inhibition. For proper enzyme-substrate reactivity, pH of the substrate buffer should be optimized. (Taylor et al., 2013.) The potency of antibody denotes specific ability of primary antibody to yield sufficiently high signal intensity in the detection of target of protein. Interestingly, extremely high concentrations of secondary antibody have been shown to inhibit the intensity of the signal achieved. Also, care must be taken when handling primary antibody to avoid its inactivation by multiple freeze-thaw cycles or long-term storage. (Snider, 2014.)

1.4 Fluorescence microscopy

Fluorescence microscopy utilizes fluorescence or phosphorescence and the principles of absorption and reflection in substances. The technique is important in both biological and medical sciences.

The structure of wide-field fluorescence microscope enables to efficiently collect fluorescent light which forms the image. The composition of the microscope makes it possible to first illuminate the sample with excitation light and then to remove the scattered signal from emitted light. A set of filters, definite for a particular wavelength, is used to obtain the final image (Demchenko, 2008). In multicolor fluorescence microscopy various fluorophores are applied onto one specimen. Multicolor images are then composed by combining several single-color images (Spring and Davidson, 2014).

1.5 Principle of immunofluorescence staining method

Immunofluorescence (IF) microscopy is a technique very similar to immunohistochemistry. The only difference is the utilization of fluorescent reporter molecules instead of chromogenic ones in the detection. There are several different possibilities of the application of fluorescent detection; for example, fluorescently labelled primary antibody, fluorescently labelled secondary antibody, and biotin-labelled secondary antibody detected with fluorescently labelled streptavidin. Furthermore, fluorescently labelled tyramides can be applied if the TSA system is used. (Odell and Cook, 2013; abcam.com.)

Fluorophores are compounds, characterized by an ability to emit light upon light excitation. They absorb light of a specific wavelength and emit it at a different wavelength. Fluorescein isothiocyanate (FITC), derivative of fluorescein, is one of the most widely used fluorophores. Other common fluorophores are derivatives of rhodamine (especially tetramethylrhodamine, i.e. TRITC), coumarin, and cyanine. (Rietdorf J, 2005.) Newer fluorophores, such as pyrene, coronene and lanthanides have been developed for brighter signal, diminished pH-sensitivity and/or to overcome the inherent problem of photo-lability of fluorophores (Tsien and Waggoner, 1995; Lakowicz, 2006), meaning the loss of signal over time with exposure to light - also called bleaching (Odell and Cook, 2013).

The problems of background staining and weak target signal are common for IHC and IF. As with IHC, the quality of the signal in IF depends on many technical details of the staining protocol, such as quality and concentrations of antibodies applied (Odell and Cook, 2013). In IF, a specific problem associated with FFPE samples is autofluorescence. It means natural emission of light by biological structures often difficult to distinguish from that of added fluorescent reporters. Tissue sample may show inherent autofluorescence or fixation method, such as aldehyde, may cause it. For example, blood vessels may show inherent autofluorescence. FFPE samples may have autofluorescence also from paraffin even after clearing. (Snider, 2014; Davis et al., 2014.) TSA method can be applied to increase particularly the signal obtained from target, thereby helping to resolve the problem of autofluorescence. However, undesirable fluorescence can be caused by nonspecific binding of dye or unbound dye. (www.thermofisher.com.)

Photobleaching also is considered as one of disadvantage of fluorescent stainings (Pawley, 2006).

It is particularly beneficial when identifying simultaneously several antigens in one specimen utilizing fluorescent labeling with several dyes. For example, Mason et al (2000) have published a method for double IF in routinely processed paraffin section and Brouns et al (2002) have described a method for triple IF staining.

1.6 Simultaneous detection of multiple antigens using immunohistochemistry and immunofluorescence

Either chromogenic or fluorescent reporters can be used in the detection of multiple antigens (Taylor et al., 2013). Robertson and coworkers (2008) have presented simultaneous detection of even three antigens in one tissue section. In double labeling a combination of two primary antibodies is used to detect two targets. Some specific problems should be taken into account and resolved in the optimization of the protocol for double staining.

First, most of the primary antibodies available are raised in mouse or rabbit, but it is often hard to find a pair of primary antibodies raised in different host species for the detection of particular targets of interest. This leads to a problem of cross reaction if indirect detection method using species specific secondary antibodies are applied instead of utilization of directly labelled primary antibodies. However, directly labelled primary antibodies are expensive and the sensitivity of the detection is low (Boorsma, 1984; Stengl and Hildebrand, 1990). Alternatively, pair of primary antibodies from same host species but of different isotype or subclass may be used. In practice, this applies mainly for mouse monoclonal primary antibodies since rabbit monoclonal antibodies are usually of IgG isotype and rabbit has only one IgG subclass, while e.g. mouse has five different IgG subclasses. (Taylor et al., 2013.) Besides to difficulties related to the availability of primary antibodies, also selection of fluorescent reporter molecules with minimally overlapping spectral characteristics, and at the same time stable and bright signal may sometimes be an issue for double immunofluorescence (Van Der Loos, 2014.). Nevertheless, many different fluorescent compounds have been launched in recent years to solve the problem (Zheng et al., 2014).

There are some specific limitations associated with double IHC in comparison to double IF. As to spectral differentiation, it is even more challenging when chromogenic reporters are in question. Especially, when the targets of interest are colocalized into the same cellular compartment, mixed colors of chromogens are hard to differentiate. The most commonly used substrate DAB, in particular, is very difficult for spectral

unmixing (van der Loos, 2008). In that case, fluorescent reporters are more convenient to use. However, an advantageous characteristic of precipitate formation is associated with DAB and utilized in double IHC when primary antibodies raised in one host species need to be applied. This means that precipitate formed by DAB-substrate shelters (Figure 5) the first primary antibody after the detection of the first target and prior to the detection of the second target. Thereby, the precipitate blocks the cross reaction between the first primary antibody applied and the secondary antibody used for the detection of the second target. (Sternberg and Joseph, 1979.) However, concentration of primary antibody may affect the result, and if used at too high concentrations, leaves the complex unsheltered (Van der Loos 2008). Additionally, sheltering is not recommended for colocalization studies (Van der Loos 2008), because one of the closely located targets may be missed by sheltering (Valnes and Brantzaeg, 1984.)

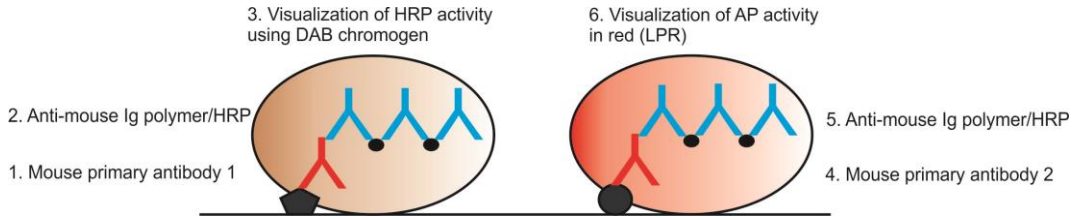


Figure 5. Principle of sheltering effect by DAB-precipitate in sequential double IHC applying two primary antibodies raised in mouse and anti-mouse secondary antibodies. Modified from Van der Loos, 2008. Precipitate formed by DAB-substrate shelters the first primary antibody after the detection of the first target and prior to the detection of the second target. Thereby, the precipitate blocks the cross reaction between the first primary antibody applied and the secondary antibody used for the detection of the second target. (Sternberg and Joseph, 1979.)

1.6.1 Double IF using primary antibodies raised in different host species

Primary antibodies raised in two different host species are commonly used in double immunofluorescence staining. Bound primary antibodies can be detected, for example, by indirect method using species-specific secondary antibodies conjugated with fluorophores. Typical protocol for FFPE tissue includes following steps (Buchwalow and Bocker, 2010; www.thermofisher.com).

1. Deparaffinization and rehydration.
2. Antigen retrieval, for example by protease digestion or in buffer (e.g. citrate buffer, pH 6) in microwave oven, autoclave or pressure cooker.
3. Blocking of unspecific reactions of antibodies by incubation in solution containing e.g. BSA, gelatin or nonfat dry milk, or in serum of animal species, in which secondary antibody was raised.
4. Primary antibodies applied simultaneously as cocktail (raised in e.g. mouse and rabbit).
5. Secondary antibodies labelled with different fluorophores applied simultaneously as cocktail (e.g. anti-mouse and anti-rabbit).
6. Counterstaining of DNA material in nuclei with DAPI.
7. Mounting.

If high signal intensity is needed, also TSA can be applied, and multicolor labelling is typically applied sequentially utilizing horseradish peroxidase labelled secondary antibodies. In that case, enzyme activity used in the detection of the first target must be totally quenched before the detection of the next one. Otherwise, false signal - derived from enzymatic activity of the first secondary antibody and of the second fluorescent tyramide substrate - can be observed. There are several possibilities for quenching the enzymatic activity of HRP described in literature, such as treatment in hydrogen peroxide or hydrochloric acid, or boiling. (www.perkinelmer.com, 2015; Roth K, 2008.)

1.6.2 Double IF using primary antibodies raised in same host species

As described above, it is often difficult to find pairs of primary antibodies suitable for diagnostic use and/or raised in different host species. In literature, different variations of immunofluorescence methods have been introduced to overcome this problem. For example, primary antibodies can be directly labelled with fluorophores (Tsirui et al., 2000) but this type of staining is not as sensitive as an indirect one and large amount of antibodies is needed. Therefore, indirect methods are in wider use. (Van der Loos 2008). One possibility is to use primary antibodies from one host species but different immunoglobulin isotypes. In this case, isotype specific secondary antibodies are utilized (Buchwalow et al., 2005). In sequential double IF the detection of the first target can be performed using a system with greater sensitivity compared to that of the latter target. In that case, the first primary antibody is applied at very low concentration, and therefore it can't be visualized in the latter detection step. That's why cross reaction

does not cause a visible problem. Furthermore, some authors describe monovalent Fab fragments to block residual binding sites on the first primary antibody prior to the application of the latter secondary antibody (Brouns et al., 2002; Lewis Carl et al., 1993).

To overcome limitations related to the availability of primary antibodies, publications report also on elution or denaturation of an antibody after the detection of the first target. In these staining methods, covalent binding of fluorescent tyramides to tissue can be employed, thus enabling usage of harsh treatments in removal of the antibodies already used. If the elution or the denaturation succeeds primary antibodies raised in one host species and of the same isotype can be used to detect the second target. Obviously, success of removal has to be monitored with proper controls. (Bauer et al., 2001.)

Toth and Mezey (2007) have described a heat-mediated stripping method of boiling in citrate buffer for the elution of rabbit primary antibody after first round of IF. Likewise, Ikeda et al (2011) have used heating in citrate buffer and found that time needed to elute different primary antibodies differs a lot and thus, time of treatment should be optimized for each antibody individually. They have reported that several antibodies can survive detectable even after 60 min treatment. That's why, the order of the application of primary antibodies can often be crucial, too. (Ikeda et al., 2011). Also, the usage of different types of treatments of the specimen in a microwave oven may be tested as long as the target to be detected after the treatment is stable under these conditions (Toth and Mezey, 2007; Lan et al 1995). Nevertheless, sometimes treatment in a microwave oven can't completely exclude the signal obtained from the first detected target. For example, study published by Bauer et al (2001) shows that desmin, ASM-1 and ki-67 antigens can't be denaturated by microwave treatment.

Various chemical treatments have also been tested for the elution of the antibodies, such as acidic solutions, like buffered glycine-hydrochloric acid and unbuffered hydrochloric acid, or buffered solution containing SDS and di-thiothreitol (Nakane, 1968). Also, a buffer containing glycine and SDS, and having low pH-value, has been proposed. (Pirici et al., 2009.) Recently, Gendusa et al (2014) published an extensive study showing that some antibodies are able to survive detectable even after application of harsh conditions such as pH 2, temperatures above 60^0 and chaotropic agents. They found that 2-Mercaptoethanol/Sodium dodecyl sulfate (2-ME/SDS) is the most efficient solution of the tested ones for the elution of antibodies leaving most antigens embedded in tissue untouched though. However, it was also shown that conditions required for successful elution depends on the affinities of the antibodies.

For some antibodies, having low affininity, usage of either acidic solution or hot chaotropic agent was enough for efficient elution, while 2-ME/SDS-treatment was required for high affinity antibodies. Furthermore, the authors state that monoclonal antibodies with high affinity may require multiple elution steps. (Gendusa et al., 2014.) Rabbit monoclonal antibodies are characterized by a higher affinity to their target epitopes compared to mouse monoclonal and rabbit polyclonal antibodies (Pope et al., 2009).

2. AIMS

This thesis concentrates on establishing a technique for the visualization of multiple antigens in human formalin-fixed paraffin embedded tissue by immunofluorescence. The specific aim is to test the applicability of tyramide signal amplification system to overcome the inherent problem of autofluorescence seen in FFPE specimens. Additional emphasis is to explore the utilization of two primary antibodies raised in one host species in multicolor imaging. Medical focus is given on selected biomarkers for colorectal and breast cancer with special attention to the EGFR-signaling for CRC and proliferation related protein securin for BC.

3. MATERIALS AND METHODS

3.1 Tissue material and reagents

Archival tissue samples from patients treated for colorectal or breast cancer were obtained from the Department of Pathology, Turku University Hospital. Additionally, lymph node tissue was used for control and optimization purposes. Specimens had been fixed in neutral buffered formalin and embedded in paraffin according to routine processing of tissue samples in the Department of Pathology. They were cut in 3-4 µm sections and placed onto SuperFrost Plus (Thermo Scientific, cat. J1800AMNZ) microscope slides. Sections were kept in an incubator overnight at 60 °C to melt paraffin and to attach tissue on slide.

Primary antibodies used in the study are described in table 1. Diluents used for the preparation of primary antibody dilutions were Antibody Diluent (Roche/Ventana, cat. 251-018), Discovery PSS Diluent (Roche/Ventana, cat. 760-212), DAKO REAL Antibody Diluent (Dako, cat. S2022) and Signal Stain (R) Antibody diluent (Cell Signaling Technology, cat. 8112S).

Table 1. List of the primary antibodies used in the study

Antibody	Type	Clone	Manufacturer
EGFR	mouse monoclonal	25	Abcam cat. ab 49716
EGFR	rabbit monoclonal	5B7	Ventana cat. 790-4347
p-mTOR 2448	rabbit monoclonal	49F9	Cell Signaling Technology cat. 2976
pAKT 473	rabbit monoclonal	EP209Y	Abcam cat. 81283
pAKT 473	rabbit monoclonal	D9E	Cell Signaling Technology cat. 4060
Vimentin	mouse monoclonal	V9	Ventana cat. 790-2917
E-cadherin	mouse monoclonal	NCH-38	DAKO cat. M3612
Cytokeratin Pan	mouse monoclonal	AE1/AE3	Invitrogen cat. 180132
Ezrin	mouse monoclonal	3C12	Olli Carpén Lab, Helsinki
Cleaved caspase 3 Asp175	rabbit monoclonal	D3E9	Cell Signaling Technology cat. 9579S
Securin	mouse monoclonal	DSC-280	Abcam cat. ab 3305
Securin	rabbit monoclonal	EPR 3240	Abcam cat. ab 79546
PTTG1IP	rabbit polyclonal	-	Abcam cat. ab 128040
ki67	rabbit polyclonal	-	Millipore AB9260

In IHC, commercial pre-formulated buffers CC1 and CC2 (Roche/Ventana, cat. 950-124 and 950-123, respectively) were used in antigen retrieval, and OmniMap anti-ms HRP (Roche/Ventana, cat. 760-4310) and OmniMap anti-rb HRP (Roche/Ventana, cat. 760-4311) multimer secondary reagents together with ChromoMap DAB substrate kit (Roche/Ventana, cat. 760-159) were used for the detection of primary antibodies. Counterstaining was performed using Hematoxylin II (Roche/Ventana, cat. 790-2208) and Bluing Reagent (Roche/Ventana, cat. 760-2037). Deparaffinization solution EZPrep (Roche/Ventana, cat. 950-102) and Reaction Buffer (Roche/Ventana, cat. 950-300) used in IHC-staining were purchased from Roche. Pertex (Histolab, cat. 00811) was applied for mounting.

In IF, antigen retrieval solutions Target Retrieval Solution pH 6.1 and Target Retrieval Solution pH 9.0 (Dako, cat. S169984-2 and S236784-2, respectively) were applied. TSA-kits 2 and 41 (Molecular Probes by Life Technologies, cat. T30954 and T20912) were used in the detection of primary antibodies and ProLong Gold antifade reagent with DAPI (Molecular Probes by Life Technologies, cat. P-36931) for mounting. IF-

stainings included also following reagents: SDS 99% (Sigma, cat. L4390), Trizma base (Fluka analytical, cat. 93352), Riboclear (Ventana, cat. 760-4125), xylene (VWR, cat. 1307), ethanol (VWR, cat. 1170), 30 % hydrogen peroxide (VWR, cat. 2014) and 2-Mercaptoethanol (Pharmacia Biotech, cat. 17-1317-01). 10x Phosphate-buffered saline (PBS) was prepared from NaCl (JT Baker, cat. 0278), KCl (Merck KGaA, cat. 4089933), Na_2HPO_4 (JT Baker, cat. 141965) and KH_2PO_4 (Merck KGaA, cat. 104873) (recipies and calculations included in the appendices).

3.2 Immunohistochemical staining

Fully automated DISCOVERY XT (Roche/Ventana) platform was utilized in visualization of expression patterns of specific biomarkers prior to application of same primary antibodies in IF-stainings. DISCOVERY XT slide staining system is computer controlled and automates the process from deparaffinization to counterstaining. It allows choice from multiple predetermined options of pretreatment conditions as well as blockers and detection systems. In this system, all incubations are performed under oil coverslip on individually heated hotplates.

In the optimization of each staining, different antigen retrieval buffers and incubation times, as well as varying dilutions, incubation times and temperatures for primary antibodies, were applied. Usually, several combinations of conditions had to be tested for optimal result. Sometimes, various incubation times for secondary antibody multimers needed to be tried, too, to increase signal obtained using certain primary antibodies (tables included in the appendices). In this study, diluted primary antibodies were added manually onto each slide during staining run applying volume of 100 µl per slide for each staining. After counterstaining slides were collected and washed using mild dishwashing liquid (Fairy Original, P&G) to remove oil coverslip. Finally, slides were manually dehydrated in series of ethanol and xylene and mounted for light microscopy.

3.3 Immunofluorescence staining and fluorescence microscopy

IF-staining was performed manually utilizing TSA-system. The basic protocol recommended by Life Technologies was followed. However, optimal dilutions of secondary antibodies and fluorescent tyramide conjugates were tested before proceeding to the optimization of double labellings. In double IF, the protocol developed for single IF was utilized applying it sequentially two times for detection of two antigens. Pairs of primary antibodies raised in either different or same host species were tested. More detailed description of different treatments applied between sequential detections has been given in the following sections. In addition, several

stainings were performed leaving out different combinations of detection reagents to control for possible autofluorescence, endogenous peroxidase activity and cross reactions. The applicability of the developed single and double IF basic protocols were further tested using several primary antibodies for different biomarkers. In these stainings, dilutions of primary antibodies were optimized for each biomarker using single IF before proceeding to double labelling.

In IF, sections of FFPE tissues were first sequentially treated with xylene and series of graduated ethanol to remove paraffin, and rinsed in distilled water. Heat mediated antigen retrieval was performed in common household microwave oven in 200 ml of buffer with specific pH value. Two different buffers (pH 6.1, 0.1x Dako and pH 9.0, 1x Dako) were used in this study. The power settings in microwave treatment were, first, 600W 7 min, and then 450W 7 min. After the treatment slides were let to cool down 20 min on the table. Quenching of the endogenous peroxidase activity was performed in freshly prepared 3% hydrogen peroxide solution. Subsequently, slides were treated with 1% BSA solution to block unspecific interactions between antibodies and tissue components. Then, primary and HRP-conjugated secondary antibodies were sequentially applied on sections following by the application of Alexa Fluor 488 or Alexa Fluor 555 tyramide substrate to visualize target. Finally, sections were counterstained and mounted with antifade reagent containing DAPI. Slides were incubated at room temperature in dark, then sealed with nail polish and stored in fridge. In the protocol described above, slides were rinsed three times with 1x PBS solution prior to the quenching of endogenous peroxidase activity and following the incubations with primary and secondary antibodies. Rinsing and quenching steps were performed in a jar in large volume of solutions. However, other incubations were carried out in humidified chamber. In that case, smaller volume of reagents, typically from 100 to 250 µl, was used depending on the size of the tissue section on the slide. Tyramide stock solution, horseradish peroxidase (HRP) conjugate stock solution and amplification buffer in H_2O_2 were prepared according to the instructions of TSA-kit. Tyramide stock solution and HRP conjugate stock solution were diluted just before use in each staining.

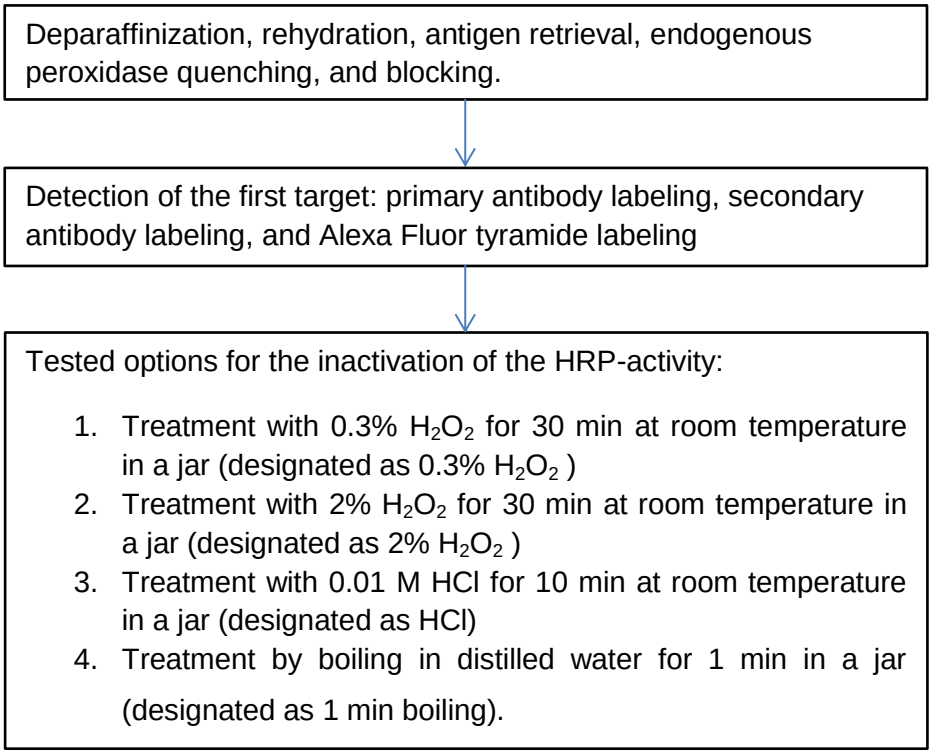
Slides were examined with wide-field microscope Leica DM RXA (upright). Three filter sets were applied for fluorescent signal detection: blue for DAPI (excitation filter BP 340-380, dichromatic mirror 400, suppression filter LP 425, Leica code A), green for Alexa488 (emission wavelengths 450-490nm) and red for Alexa555 (emission wavelengths 570-640nm). Images were saved using Nikon Camera Control Unit DS-Fi2.

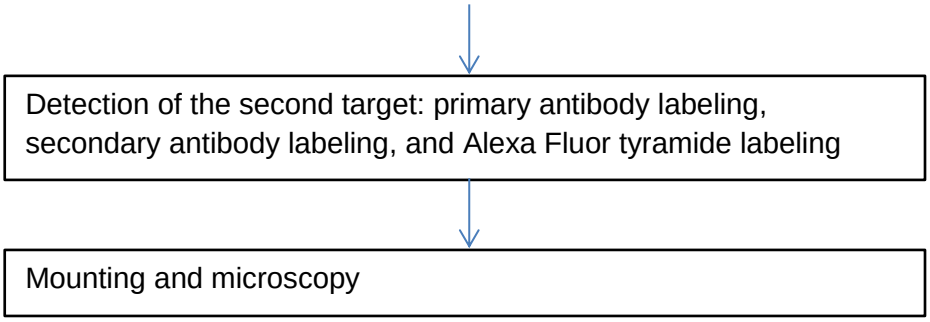
Summary of single-IF protocol for FFPE tissue specimens:

1. Overnight incubation at +60 °C
2. Deparaffinization
3. Rehydration in series of xylene and descending ethanols, finally to distilled water
4. Antigen retrieval
5. Endogenous peroxidase quenching, 60 min at RT
6. Blocking, 60 min at RT
7. Primary antibody labeling, 60 min at RT
8. Rinsing 3 times with 1xPBS
9. Secondary antibody labeling, 60 min at RT
10. Rinsing 3 times with 1xPBS
11. Alexa Fluor tyramide labeling, 10 min in dark at RT
12. Rinsing 3 times with 1xPBS
13. Mounting

3.3.1 Double IF using primary antibodies raised in different host species

Double IF using primary antibodies raised in different host species was performed sequentially combining two pre-optimized single IF stainings. Schematic diagram below describes a general outline of the protocol used and the different conditions tested.





3.3.2 Double IF using primary antibodies raised in same host species

Double IF using primary antibodies raised in same host species was performed sequentially as described in the schematic diagram included in the previous section. However, types of treatments applied between the detections of targets differed from those represented previously. In this case, a specific purpose was to elute or to denaturate the primary antibody which has already been detected – along with inactivation of HRP-activity – in order to avoid cross reaction between the first primary and the second secondary antibody. In this study, following combination of treatments were applied between the detections of the first and the second target:

1. Boiling for 1 min in distilled water (designated as 1 min boiling)
2. Boiling for 3 min in distilled water (designated as 3 min boiling)
3. Treatment with Riboclear for 10 min at 37 °C, and rinsing 3 times with 1xPBS (designated as Riboclear)
4. Treatment with Riboclear for 10 min at 37 °C, and rinsing 3 times with 1xPBS followed by boiling in distilled water for 3 min, and rinsing 3 times with 1xPBS (designated as Riboclear and short boiling)
5. Treatment with Riboclear for 10 min at 37 °C, and rinsing 3 times with 1xPBS, followed by treatment with 3% H_2O_2 for 30 min at room temperature, and rinsing 3 times with 1xPBS (designated as Riboclear and H_2O_2)
6. Treatment with Riboclear for 10 min at 37 °C, and rinsing 3 times with 1xPBS, followed by heating for 10 min in distilled water at 98 °C, and cooling down for 10 min at room temperature (designated as Riboclear and heating 98 °C)
7. Treatment with 2-ME/SDS solution (0.8%/20%, v/v) for 30 min at 56 °C, and rinsing 3 times with 1x PBS (designated as 2-ME/SDS)
8. Treatment with 2-ME/SDS solution (0.8%/20%, v/v) for 30 min at 56 °C, and washing five times with 1xPBS for 5 min each (extensive washing included in order to avoid strong fluorescence from SDS) (designated as 2-ME/SDS 56 °C and PBS washing)

9. Treatment with 2-ME/SDS solution (0.8%/20%, v/v) for 30 min at 56 °C, rinsing three times with 1xPBS, and incubation in 2,5% (v/v) Triton X-100 for 10 min (designated as 2-ME/SDS 56 °C and Triton X-100 washing)
10. Treatment with 1x citrate buffer pH 6.1 (Dako, cat. S169984-2) in a house hold microwave oven (power setting 850W for 2.5 min, then 450W for 5 min) and cooling down for 30 min at room temperature), followed by rinsing 3 times with 1x PBS (designated as microwave treatment).

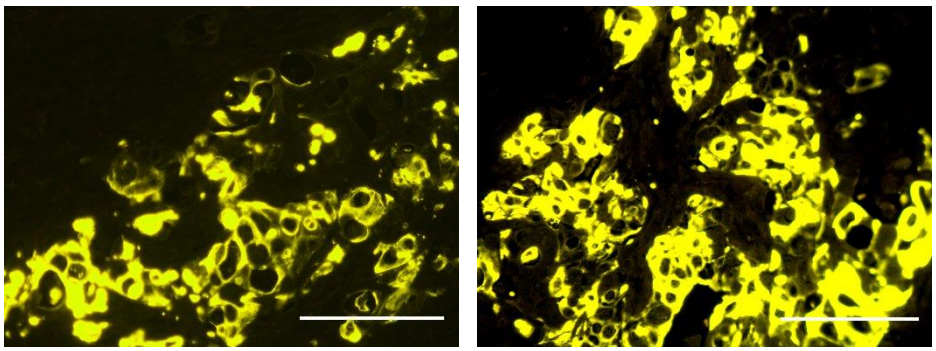
4. RESULTS

4.1 Optimization of IF staining for FFPE tissue

Tables 7 and 8 summarize all experiments accomplished for the optimization of IHC and single IF protocols for the studied 11 biomarkers. All optimized protocols for single-IF are presented in Table 10. The main idea of the optimization process of IF was, first, to develop basic protocol for single IF which could then be applied for different biomarkers, and secondly, optimize pretreatment procedures of tissue and dilutions of primary antibodies for individual biomarkers using the optimized basic IF protocol. The staining pattern seen in IF was considered reliable when it resembled that obtained from IHC.

4.1.1 Optimization of single IF

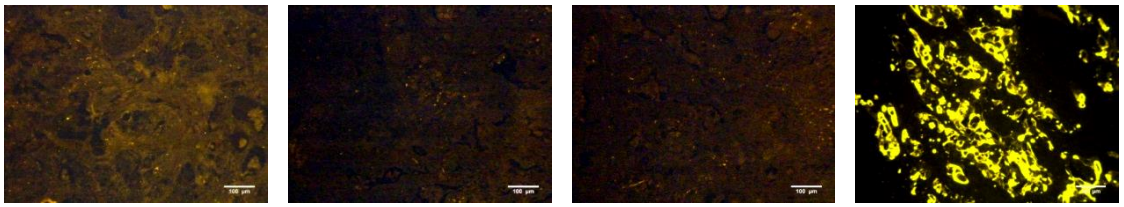
In order to optimize basic protocol for single IF, two different dilutions of secondary antibody and tyramide conjugate were tested (1:50 and 1:100). In these tests, p-mTOR primary antibody was used as an example. For further experiments, dilution 1:100 was chosen as optimal for both parameters. Figure 6 shows equal staining of epithelial cells in CRC tissue.



A B

Figure 6. Equal staining in two protocols for p-mTOR. Different dilutions of secondary antibody and tyramide were tested: 1:50/1:50 (A) and 1:100/1:100 (B). Scale bar - 100 µm.

To validate the specificity of the signal obtained from single-IF several controls were used: 1) without primary antibody, 2) without primary and secondary antibodies, and 3) without primary and secondary antibodies as well as fluorescent tyramide conjugate. In each control experiment, omitted reagent was substituted with corresponding diluent used in that step of detection. With these tests one can evaluate source of unspecific signal possibly detected – cross reactions of detection reagents, insufficient blocking of endogenous peroxidase activity in tissue and/or autofluorescence. In this study, most of the tested options showed absence of signal indicating that the protocol resulted in specific labeling. However, autofluorescence of connective tissue was often observed. In epithelial cells – the primary interest in this thesis – autofluorescence was never seen. (Figures 7 and 8).



A B C D

Figure 7. Control stainings with omitted primary antibody (A), primary and secondary antibodies (B), and primary and secondary antibodies as well as fluorescent tyramide (C). Single IF including p-mTOR primary antibody and all the detection reagents is shown for comparison (D). No signal from cross reactions, endogenous peroxidase activity or autofluorescence is seen. Scale bar - 100 µm.

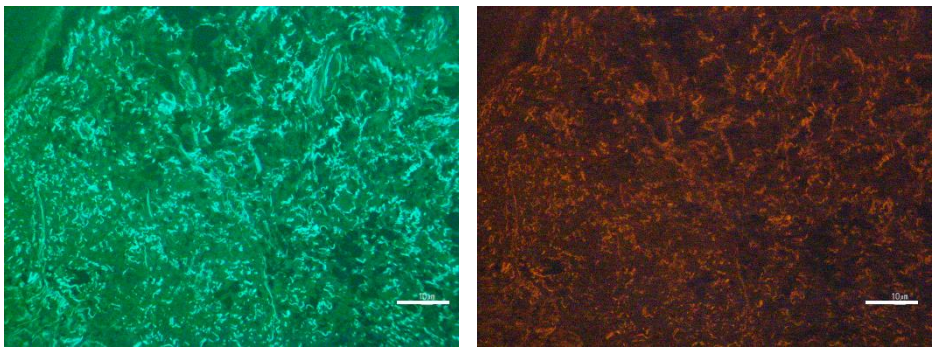
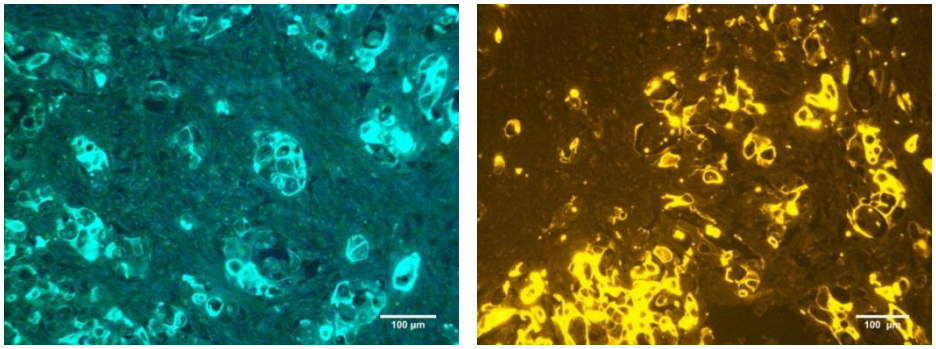


Figure 8. Autofluorescence of connective tissue was seen in both green and yellow channels. CRC sample was used as an example and protocol was performed omitting primary antibody, secondary antibody and tyramide. Scale bar - 100 µm.

In addition, two different Alexa labels were tested for immunofluorescent detection in order to compare the signal intensity obtained. p-mTOR primary antibody was used as an example (Figure 9). The applicability of the protocol was further tested using different type of tissue sample and another primary antibody. Figure 10 shows proliferating cells labelled in yellow stained with ki67 primary antibody in human lymph node tissue.



A B

Figure 9. Comparison of staining intensities obtained by using Alexa488, green (A) and Alexa555, yellow (B) shows comparative signal in CRC tumor epithelial cells in single IF using p-mTOR primary antibody as an example. Scale bar - 100 µm.

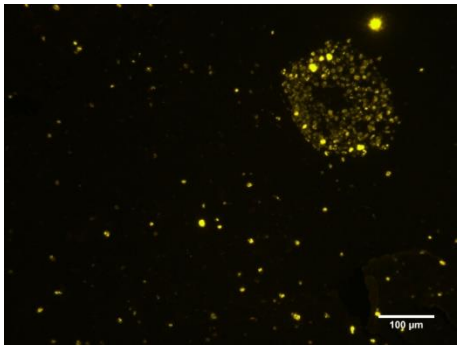


Figure 10. Single IF using ki67 primary antibody shows proliferating cells in yellow in lymph node tissue. Scale bar - 100 µm.

TSA is considered as highly sensitive method. To compare sensitivities between detection methods used in IHC and IF, differencies in the dilutions of primary antibodies needed to obtain clear signal were contrasted. As expected, higher dilutions could be used when applying TSA. For example, 1:200 dilution was required for rabbit securin and PTTG1IP primary antibodies and 1:1000 for Ezrin when detected by IHC while considerably higher dilutions 1:1000, 1:1500 and 1:20000 could be used in IF for the same antibodies, respectively (not shown).

Some of the fluorescently labelled slides sealed with nail polish were stored in dark at +4 °C and the signal intensity of the stainings was re-evaluated after one year storage. The intensity was found bright even after long storage (Figure 11).

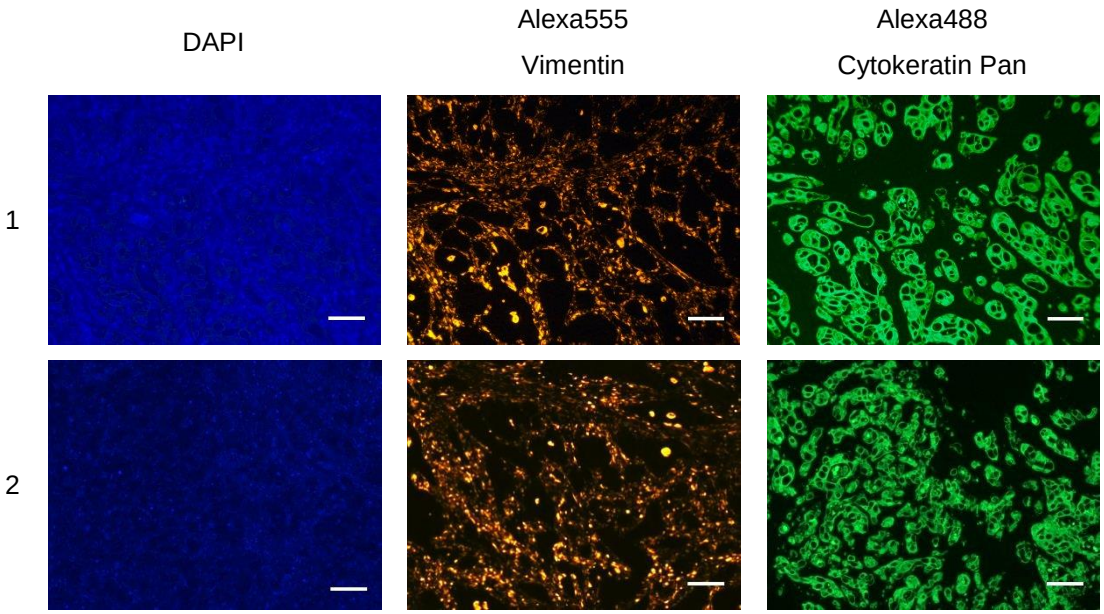


Figure 11. Signal intensity of different fluorescent labels before (1) and after 1 year of storage (2). Alexa555 (yellow) and Alexa488 (green) still showed high signal intensity. DAPI, however, was fainted over time. Scale bar - 100 µm.

4.1.2 Optimization of double IF using primary antibodies raised in different host species

Double IF can be performed sequentially, meaning that after the detection of the first antigen residual peroxidase activity from the first secondary antibody is destroyed, and then the second antigen is detected. When the applied primary antibodies are raised in different host species cross reaction between the first primary antibody and the second secondary antibody is not an issue. However, the complete inactivation of peroxidase acitivity is a requirement. In this thesis, sequential double IF was applied and four

different treatments were tested in order to test the completeness of the inactivation of HRP. Of the tested treatments, 0.3% H_2O_2, 2% H_2O_2 and HCl were not sufficient to inactivate HRP activity completely. However, 1 min boiling in distilled water was enough to inactivate HRP activity and was, therefore, applied in all double IF stainings when primary antibodies were raised in different host species. Figure 12 represents results of the tests using p-mTOR as an example. In these tests, p-mTOR primary antibody was first detected with Alexa555 (yellow), and then HRP was inactivated. After this, second primary antibody was substituted with antibody diluent and detected with Alexa488 (green). Thus, green signal seen in epithelial cells stems from the incompleteness of the inactivation of peroxidase.

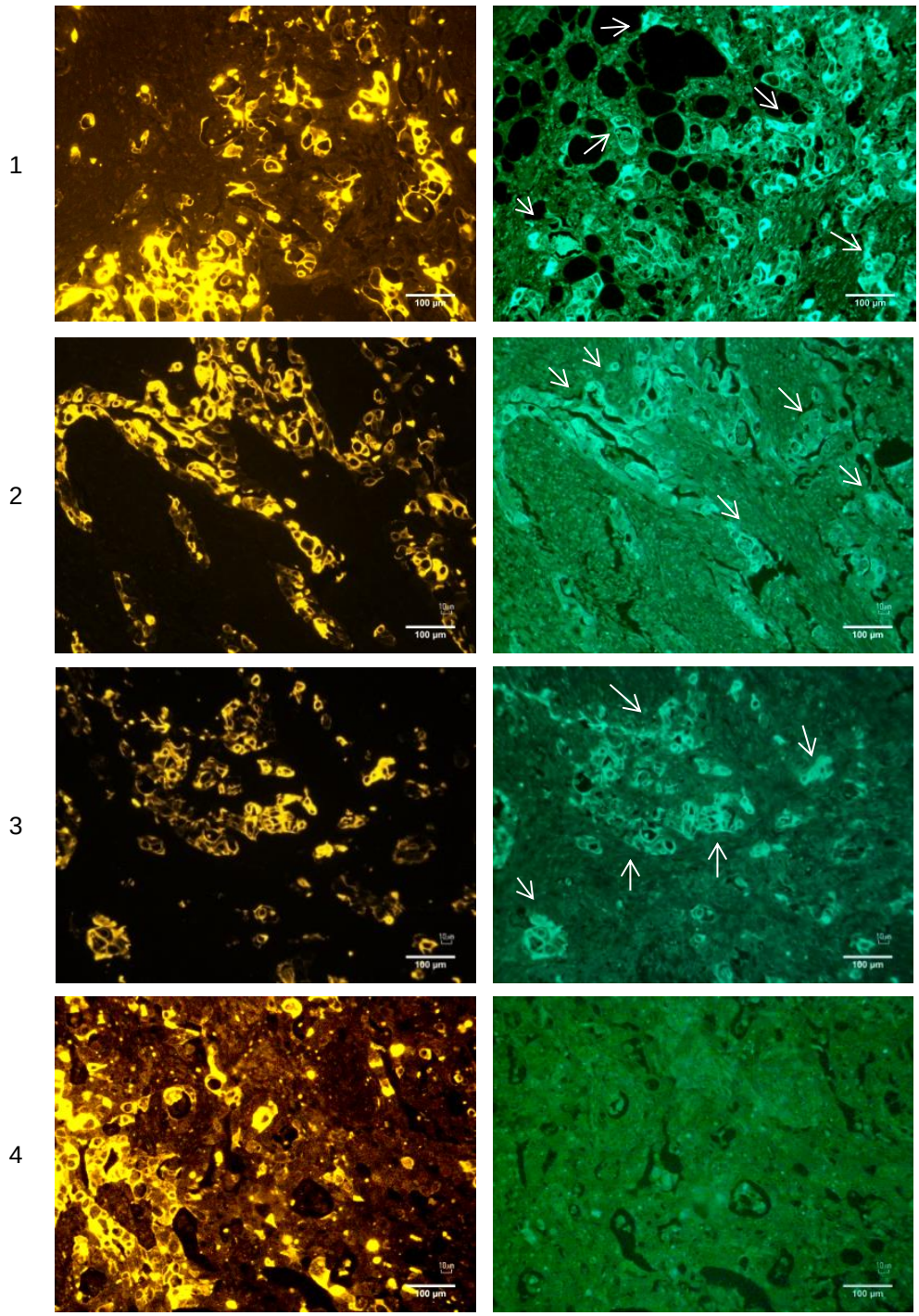


Figure 12. Different methods of inactivation of horseradish peroxidase in sequential double immunofluorescence using staining of p-mTOR in CRC sample as an example. p-mTOR was detected first (Alexa555, yellow) and second primary antibody was substituted with antibody diluent (Alexa488, green).

1, 2 and 3. Insufficient inactivation of HRP activity (0.3% H_2O_2, 2% H_2O_2 and HCl, respectively) shown with arrows.

4. One minute boiling inactivated HRP totally, no signal is observed.

Scale bar - 100 µm.

The one minute boiling protocol was chosen for further use and some controls were applied to check performance of the developed protocol: 1) omitting primary antibody in the second round of detection, 2) omitting all primary and secondary antibodies as well as tyramide conjugate in the first round of detection but including tyramide Alexa555 in the second round and 3) omitting both primary and secondary antibodies as well as tyramide conjugates both in the first and the second rounds of detection. Using these controls possible residual HRP activity, cross reactions of antibodies, incomplete quenching of endogenous peroxidase activity and autofluorescence can be evaluated. All controls demonstrated efficacy of the method since inappropriate signal was not detected, except for autofluorescence of connective tissue. Figure 13 shows an example of control staining performed omitting primary and secondary antibodies as well as tyramide conjugates.

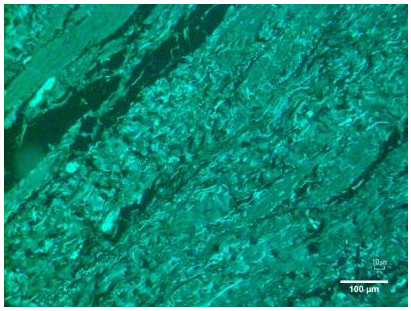
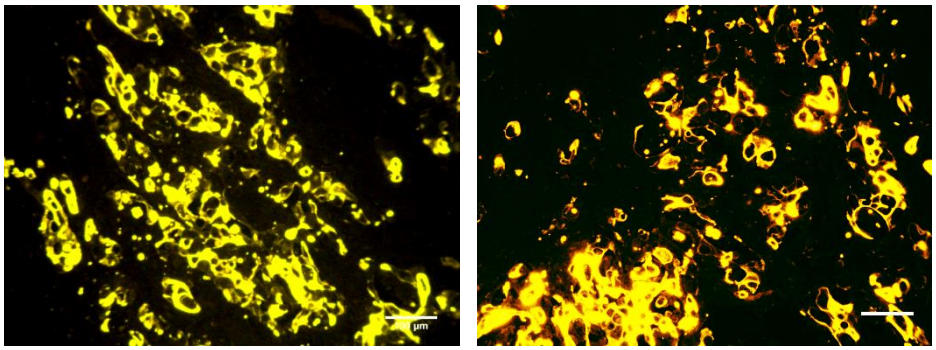


Figure 13. Control for double IF using one minute boiling between the rounds of detection. No signal was detected when primary and secondary antibodies and tyramide conjugates were omitted, except for autofluorescence of connective tissue in CRC sample. Scale bar - 100 µm.

In order to test if the signal is affected by boiling, intensity obtained by single IF was compared to that remaining after boiling. The signal intensities were comparable as shown in figure 14 using p-mTOR labelled with Alexa555 as an example.



A B

Figure 14. Signal intensity obtained from p-mTOR single IF (A) was comparable to that seen after one minute boiling in double IF (B). Scale bar - 100 µm.

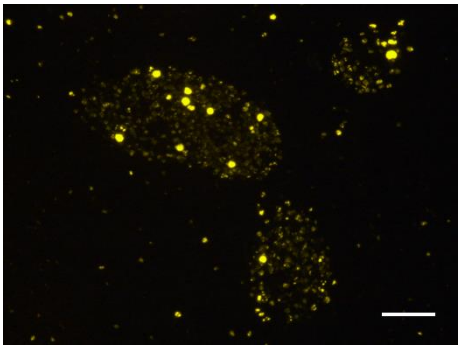
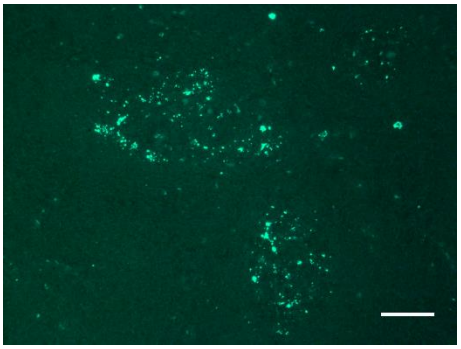
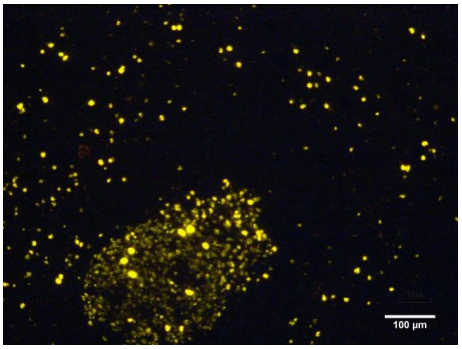
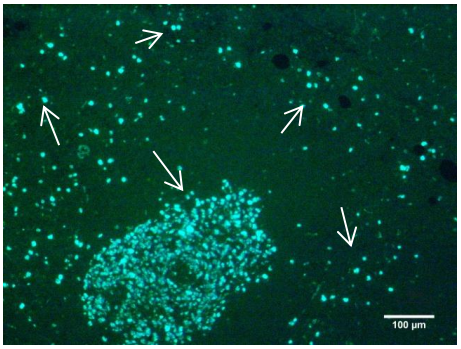
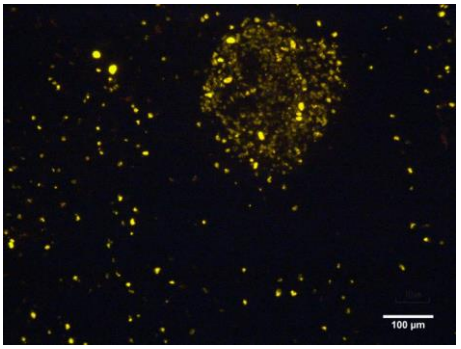
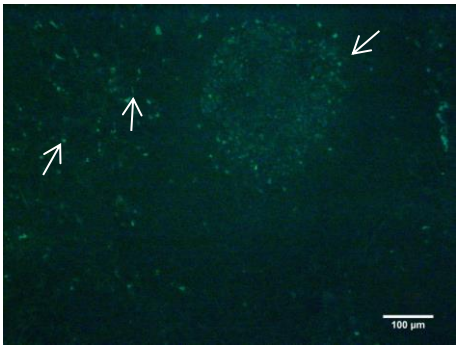
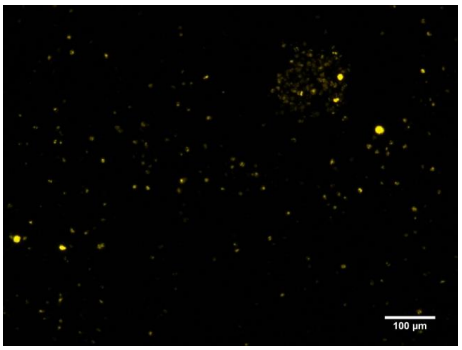
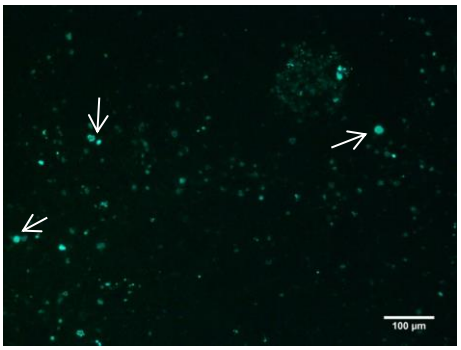
4.1.3 Optimization of double IF using primary antibodies raised in same host species

To study the possibility to use a pair of antibodies raised in same host species several sequential stainings using ki67 and Cleaved caspase 3 primary antibodies were performed. Ki67 is a proliferation marker and Cleaved caspase 3 is a marker of apoptosis, and therefore, they should not show same expression pattern – unless reaction occurs between the primary antibody applied in the first and the secondary antibody applied in the second round of detection. For some experiments, Cleaved caspase 3 primary antibody was substituted with antibody diluent. In this case, no signal from tyramide conjugate applied in the end of sequential double IF should be seen. Intermediate steps executed in order to elute / denaturate antibodies used in the detection of the first antigen, and thus applied between the sequential detections of antigens are shown in Table 2. This table includes also an evaluation of efficiency of these treatments. Results are presented in figure 15.

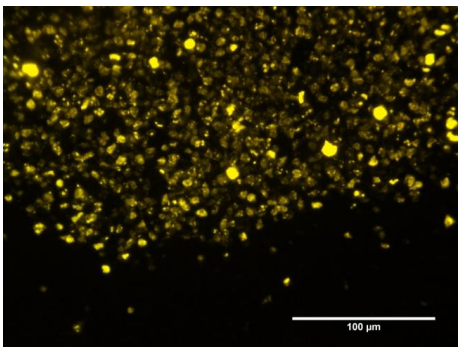
Table 2. Intermediate steps tested in double IF applying primary antibodies raised in same host species and their efficiency.

Treatment	**Efficiency**
1 min boiling	low
3 min boiling	low
Riboclear	low
Riboclear and short boiling	low
Riboclear and H_2O_2	low
Riboclear and heating 98 °C	promising
2-ME/SDS	background autofluorescence
2-ME/SDS 56 °C and PBS washing	background autofluorescence
2-ME/SDS 56 °C and Triton X-100 washing	promising
Microwave treatment	low

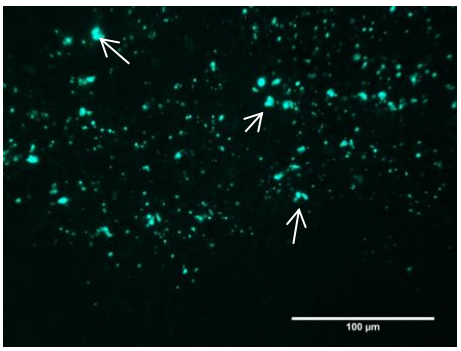
Because SDS-containing solution caused heavy background fluorescence, two methods were tested in order to remove this fluorescence from specimen – treatment with Triton X-100 for 10 min and washing five times with PBS (5 min for each). Of the tested methods, Triton X-100 removed background fluorescence better than extensive washing with PBS (Figure 15).

	Alexa555	Alexa488
1		
	ki67	Cleaved caspase 3
2		
	ki67	Antibody diluent
3		
	ki67	Antibody diluent
4		
	ki67	Cleaved caspase 3

5

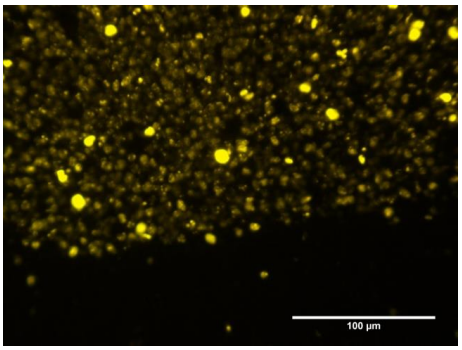


ki67

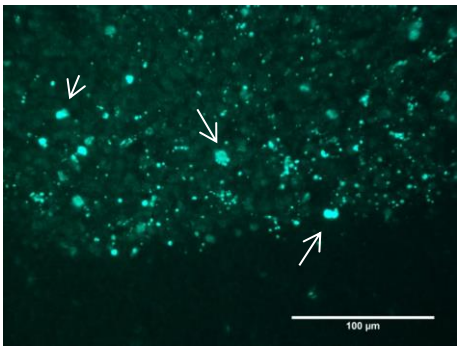


Cleaved caspase 3

6

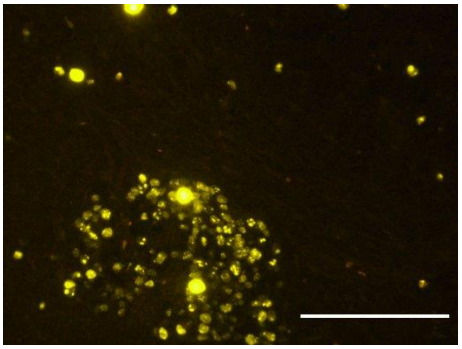


ki67

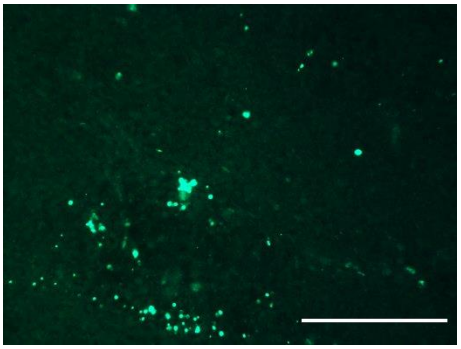


Cleaved caspase 3

7

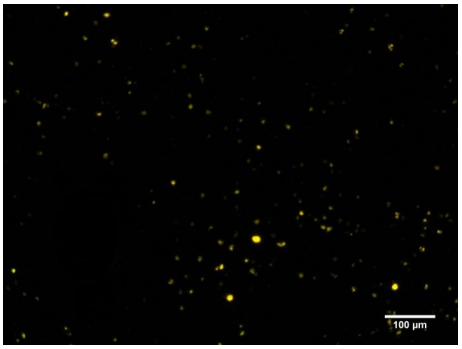


ki67

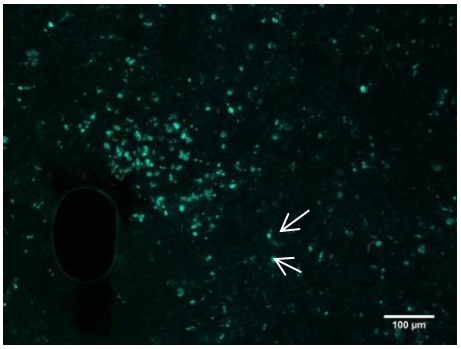


Cleaved caspase 3

8



ki67



Cleaved caspase 3

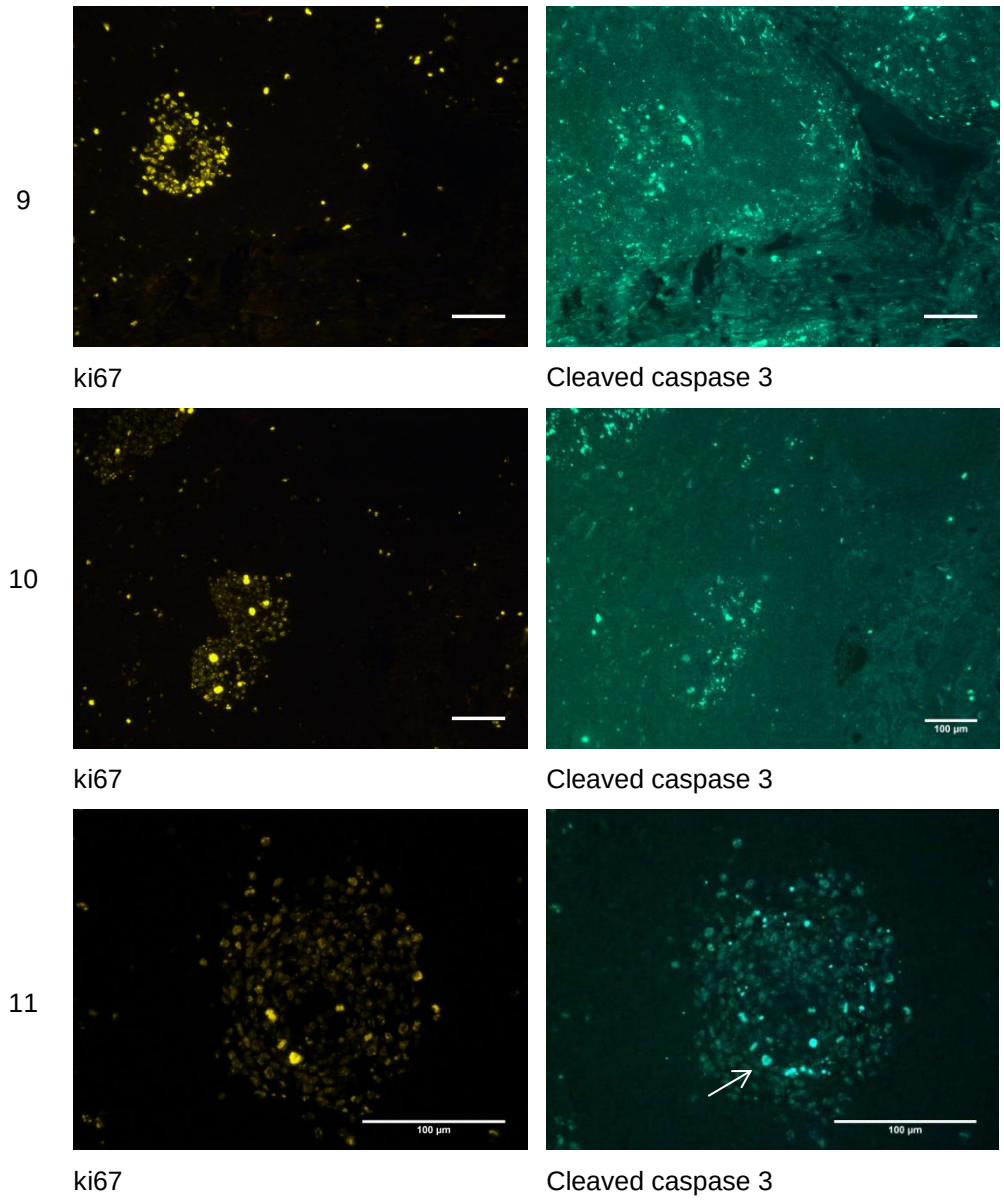


Figure 15. Staining patterns obtained from sequential double IF applying different methods for the inactivation of HRP and denaturation / elution of the primary antibody used in the detection of the first antigen. Identically stained areas shown with arrows reveal an undesirable reaction between the primary antibody applied in the first and secondary antibody applied in the second round of detection. In each experiment, ki67 was applied in the first round of detection and labelled with Alexa555 (yellow). Scale bar - 100 µm.

1. Staining patterns for ki67 and Cleaved caspase 3 demonstrated by single IF
2. 1 min boiling
3. 3 min boiling
4. Riboclear
5. Riboclear and short boiling
6. Riboclear and H_2O_2
7. Riboclear and heating 98 °C
8. 2-ME/SDS. Artefact is an air bubble.

9. 2-ME/SDS 56 °C and PBS washing
10. 2-ME/SDS 56 °C and Triton X-100 washing
11. Microwave treatment

Because treatment with Riboclear following by heating at 98 °C showed promising results, it was studied further using another pair of primary antibodies raised in same host species. Cytokeratin Pan and Vimentin were chosen for testing. Cytokeratin Pan labels epithelial cells while Vimentin labels mesenchymal cells. Double IF using Riboclear and heating 98 °C, and applying Cytokeratin Pan and Vimentin showed promising results when Vimentin was applied in the first round of double IF. Table 3 describes the tested options and the results obtained.

Table 3. Double IF applying Cytokeratin Pan and Vimentin primary antibodies and treatment with Riboclear and heating 98 °C to denaturate / elute the first primary antibody.

	1	2	3
1st primary antibody	Cytokeratin Pan	Vimentin	Vimentin
1st fluorescent label	Alexa488	Alexa488	Alexa555
2nd primary antibody	Vimentin	Cytokeratin Pan	Cytokeratin Pan
2nd fluorescent label	Alexa555	Alexa555	Alexa488
Result	Staining from Cytokeratin Pan and Alexa555	No staining from Vimentin and Alexa555	No staining from Vimentin and Alexa488

Next, double IF including the treatment with Riboclear and heating at 98 °C was performed substituting the second primary antibody with antibody diluent, and thus staining with the second tyramide conjugate should not show any labelling. Different pairs of primary antibodies were applied in these tests. Each pair of tested antibodies was raised in same host species. These tests included mouse monoclonal (Cytokeratin Pan, Vimentin, E-cadherin) and rabbit monoclonal (Cleaved caspase 3) antibodies. Of the tested antibodies, Cytokeratin Pan and Cleaved caspase 3 could not be removed with the applied treatment. However, Vimentin and E-cadherin were removed totally. (Figure 16, Table 4).

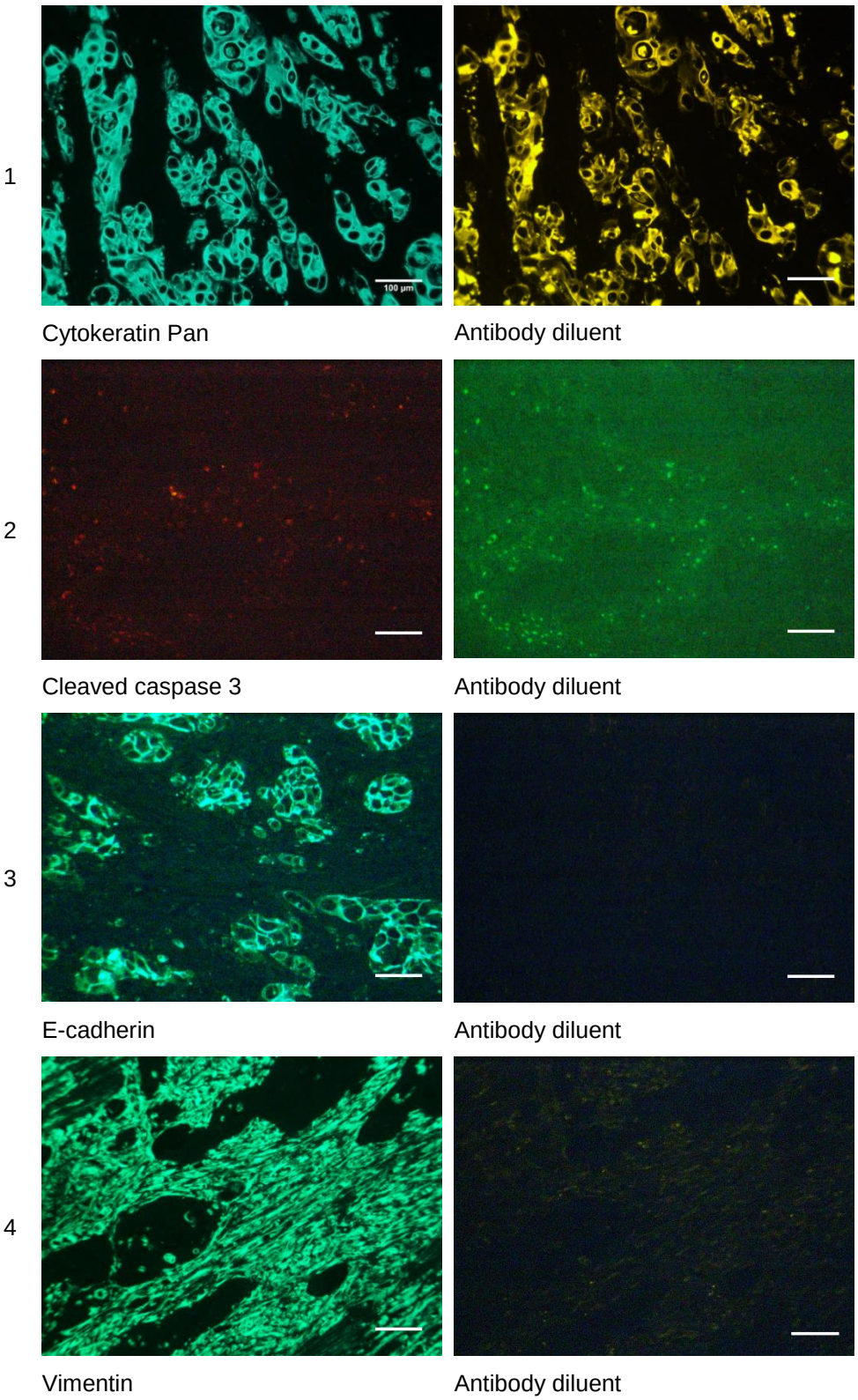


Figure 16. Double IF applying different types of primary antibodies and treatment with Riboclear and heating at 98 °C to denaturate / elute the first primary antibody. The second primary antibody applied was substituted with antibody diluent.

1. Cytokeratin Pan: epithelial cells should be seen only in Alexa488 (green), however reaction is observed also in Alexa555 (yellow) demonstrating incomplete removal of the first applied primary antibody.

2. Cleaved caspase 3: lymphocytes undergoing apoptosis should be seen only in Alexa555 (yellow), however reaction is observed also in Alexa488 (green) demonstrating incomplete removal of the first applied primary antibody.
3. E-cadherin: epithelial cells are seen only in Alexa488 (green) demonstrating total removal of the first applied primary antibody.
4. Vimentin: connective tissue is seen only in Alexa488 (green) demonstrating total removal of the first applied primary antibody.
Scale bar - 100 µm.

Table 4. Success of the removal of the different types of primary antibodies using Riboclear and heating at 98 °C.

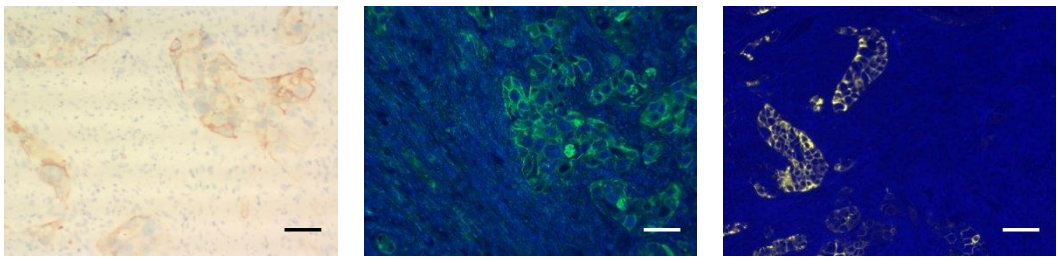
Primary antibody	**Type of the primary antibody**	**Success of the removal**
Cytokeratin Pan	mouse monoclonal	primary antibody not removed
Cleaved caspase 3	rabbit monoclonal	primary antibody not removed
Vimentin	mouse monoclonal	good, primary antibody removed totally
E-cadherin	mouse monoclonal	good, primary antibody removed totally

4.2 Application of IF for the detection of selected biomarkers in CRC

All tested conditions to optimize single IF for selected biomarkers in CRC are presented in table 9 and the best ones in table 10 in the appendices. For each primary antibody, the antigen retrieval procedure and the dilution was optimized separately. However, for some antibodies other variables needed additional testing.

4.2.1 EGFR IHC and single IF

EGFR signaling is considered to play an important role in CRC and it was chosen as the main target in this thesis. Two different EGFR antibodies – one raised in mouse and the other in rabbit – were used enabling combinations with both rabbit and mouse primary antibodies in double IF. Examples of expression patterns from different labellings using different EGFR primary antibodies demonstrate corresponding staining of epithelial cells in CRC tissue (Figure 17).



A B C

Figure 17. Different methods of detection and different EGFR primary antibodies in CRC samples.

A. IHC applying EGFR (mouse monoclonal, clone 25) primary antibody

B. single IF applying EGFR (mouse monoclonal, clone 25) and overnight incubation of primary antibody

C. single IF applying EGFR (rabbit monoclonal, clone 5B7)

Scale bar - 100 µm.

Special optimization process was required for EGFR (clone 25) primary antibody to obtain sufficiently bright signal in single IF. Different combinations of dilutions for primary antibody, secondary antibody and fluorescent tyramide conjugate were tested in the optimization process (Table 5).

Table 5. Optimization of EGFR (clone 25) single IF protocol.

EGFR (clone 25)	1:50	1:25	1:25
Secondary antibody	1:50	1:100	1:50
Alexa488	1:50	1:100	1:50

Of the tested combinations, 1:25 (primary antibody) / 1:50 (secondary antibody) / 1:50 (Alexa488) gave the best signal intensity, and thus, was chosen for further immunofluorescence experiments. To further intensify the signal, incubation time of the primary antibody was increased from one hour incubation at RT to overnight incubation in the fridge at 4 °C.

Double IF was used to check the similar expression patterns obtained by mouse and rabbit EGFR primary antibodies. Different order of primary antibodies and different fluorescent dyes were tested. For mouse EGFR (clone 25) primary antibody overnight incubation in the fridge was used. (Table 6 and figure 18). Staining patterns were similar.

Table 6. Optimization of application order for mouse and rabbit EGFR antibodies.

Experiment	1	2
First primary antibody	EGFR (clone 5B7)	EGFR (clone 25)
First secondary antibody	1:100	1:50
First tyramide	Alexa555,1:100	Alexa488, 1:50
Second primary antibody	EGFR (clone 25)	EGFR (clone 5B7)
Second secondary antibody	1:50	1:100
Second tyramide	Alexa488, 1:50	Alexa555, 1:100

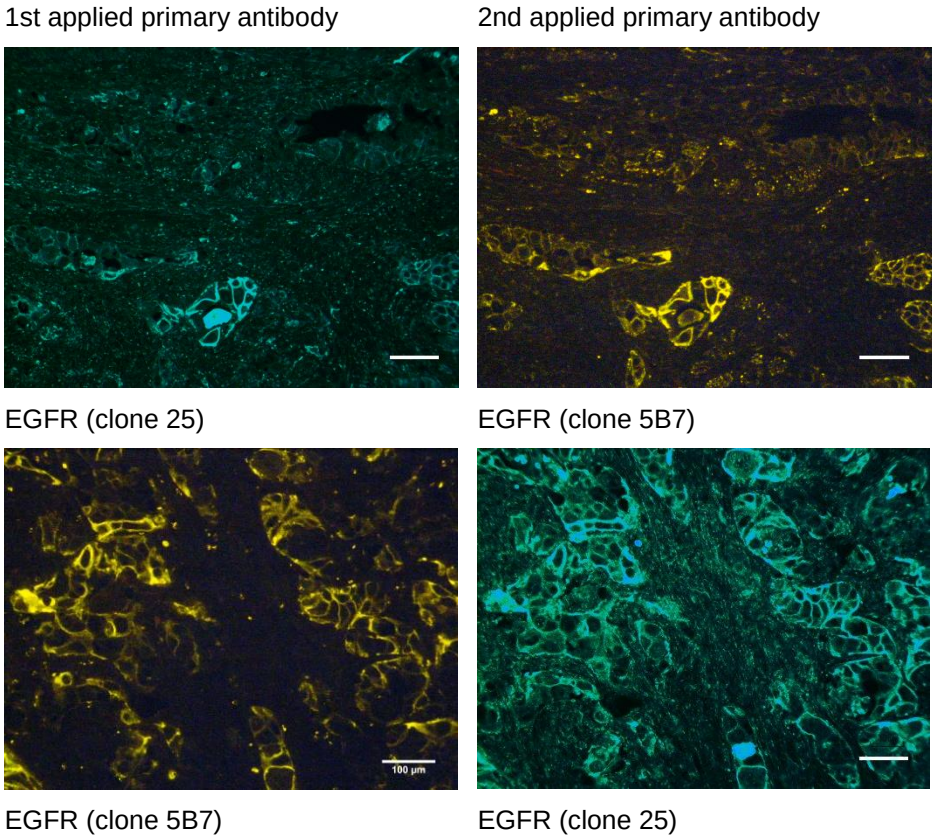


Figure 18. Double IF using EGFR (clone 5B7, rabbit) and EGFR (clone 25, mouse) primary antibodies. Application of EGFR (clone 25) in the second round of detection with Alexa488 and overnight incubation at 4 °C showed the brightest signal. Scale bar - 100 µm.

4.2.2 p-mTOR IHC and single IF

Special antibody diluents are recommended for the detection of phosphorylated proteins. Thus, labelling with p-mTOR primary antibody was tested using four different antibody diluents: Discovery PSS Diluent, 1% BSA in Discovery PSS Diluent, Antibody Diluent by Ventana and DAKO REAL Antibody Diluent. From these, 1% BSA in Discovery PSS Diluent showed the highest signal intensity in IHC and was chosen for further experiments applying IF. Examples of expression pattern of p-mTOR

demonstrated in CRC tissue both by IHC and single IF (Figure 19). Staining patterns are similar.

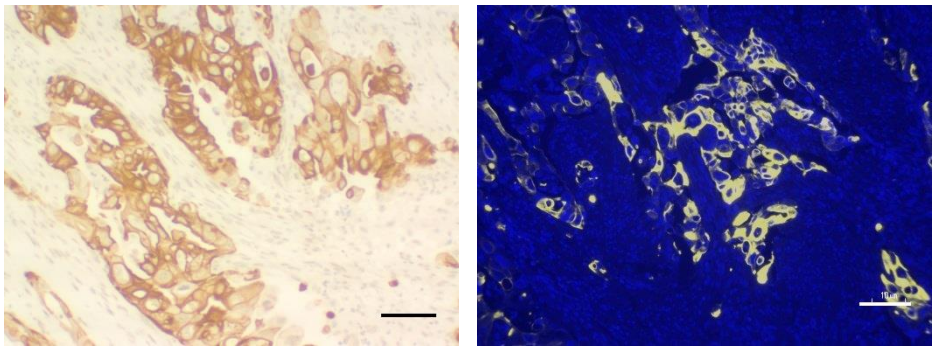
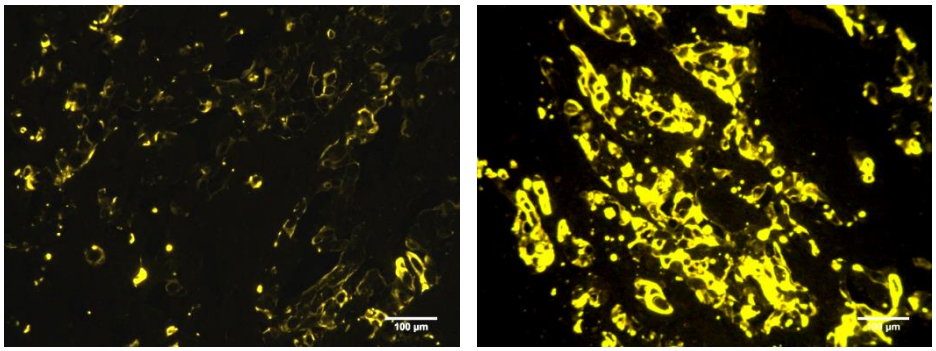


Figure 19. p-mTOR is expressed in colorectal cancer cells. Scale bar - 100 µm.

An example of drastic difference in signal intensity in p-mTOR staining after the pretreatment using either pH 6.0 or 9.0 buffer depicted in Figure 20.



A B

Figure 20. Pretreatment using antigen retrieval buffer pH 6.0 (A) showed weak signal intensity in comparison with pH 9.0 (B) in CRC sample using p-mTOR primary antibody. Scale bar - 100 µm.

4.2.3 pAKT IHC and single IF

Two different primary antibodies were tested in the detection of pAKT, since the weak signal intensity obtained in IHC. Multiple attempts were performed to optimize chromogenic staining using pAKT (phospho S473, clone EP209Y) primary antibody in CRC and BC tissue (Table 7 in appendices), but they all were characterized by strong background staining or weak target signal intensity. Another antibody – pAKT (473, clone D9E) – was applied in single IF according to the recommendation. Different incubation times and temperatures needed to be tested for IHC and single IF using this antibody in order to intensify the signal obtained. Breast cancer tissue biopsy was used in these tests in order to avoid problems related to the detection of phosphoprotein in larger tissue samples. Figure 21 demonstrates an example of pAKT staining in BC sample using primary antibody clone D9E. IF staining showed weak signal intensity.

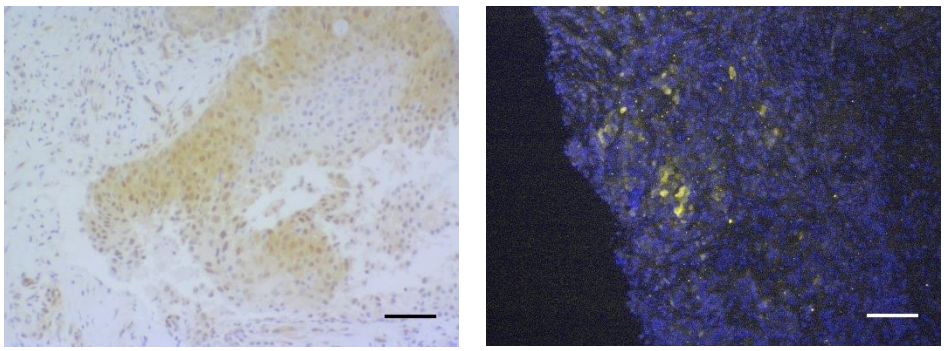


Figure 21. pAKT expression in BC tissue labelled by clone D9E in IHC (A) and single IF (B). Scale bar - 100 µm.

4.2.4 Ezrin IHC and single IF

The expression pattern obtained by Ezrin primary antibody is demonstrated in figure 22. IHC and IF provided identical staining pattern.

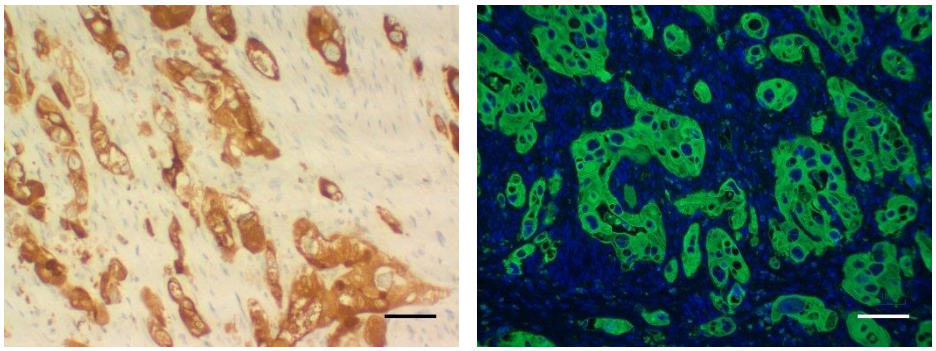


Figure 22. Ezrin is shown to be expressed in tumor epithelial cells in CRC. Scale bar - 100 µm.

4.2.5 Double IF of selected biomarkes in CRC

Expression patterns of selected biomarkers obtained by applying double IF and different combinations of primary antibodies in CRC tissue are shown in figure 23. Strong Ezrin and strong EGFR cells show stronger p-mTOR staining. EGFR expression lead to stronger Ezrin expression.

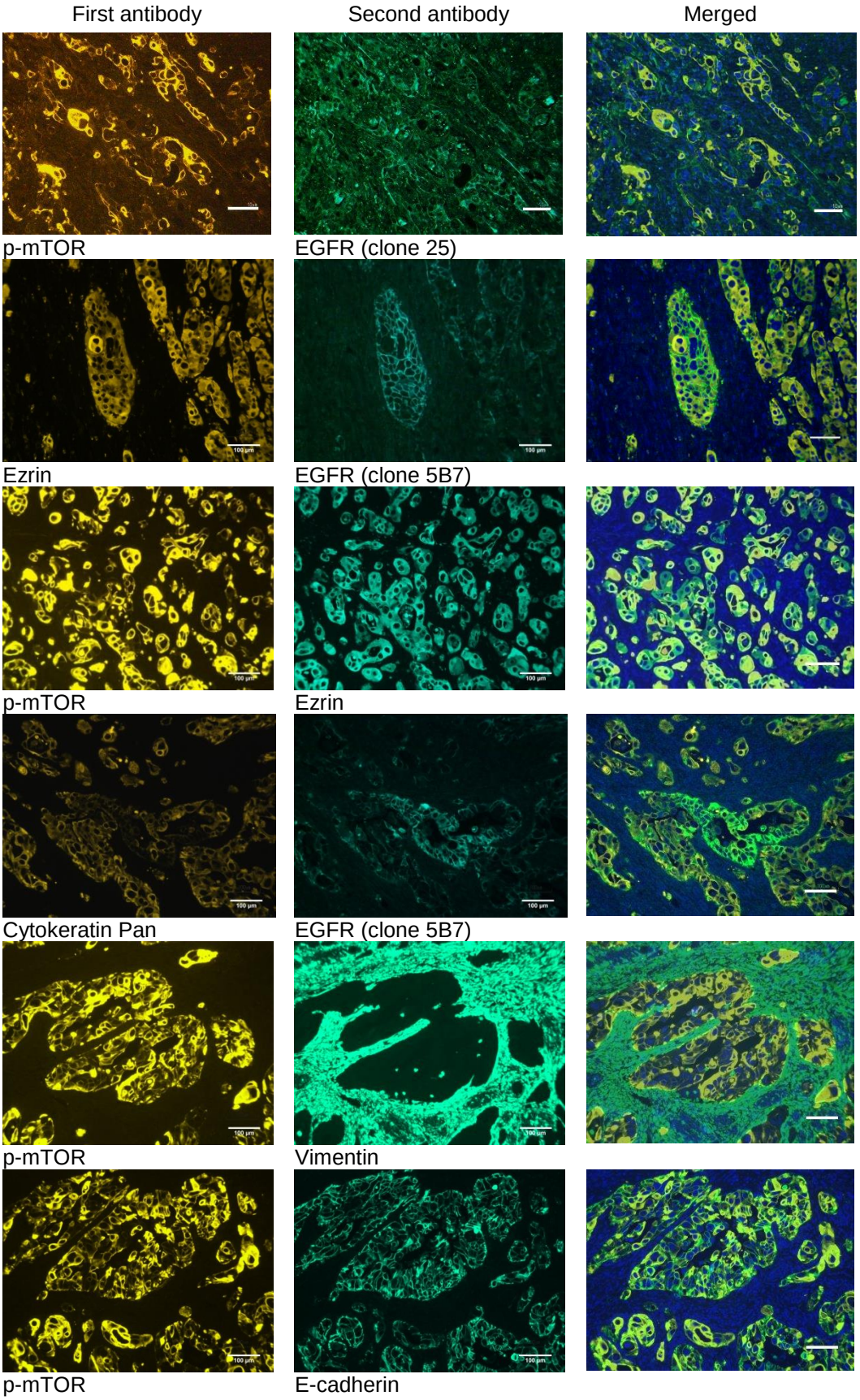


Figure 23. Double IF of selected biomarkers in CRC samples. Scale bar - 100 µm.

4.3 Application of IF for the detection of selected biomarkers in BC

All tested conditions to optimize single IF for selected biomarkers in BC are presented in table 8 and the best ones in table 9 in the appendices. For each primary antibody, the antigen retrieval procedure and the dilution was optimized separately.

4.3.1 Securin IHC and single IF

Proliferation is a corner stone in breast cancer pathology. In this study, proliferation related protein securin was studied. Two different securin antibodies were tested in order to assure similar expression pattern in different cell types and cell compartments.

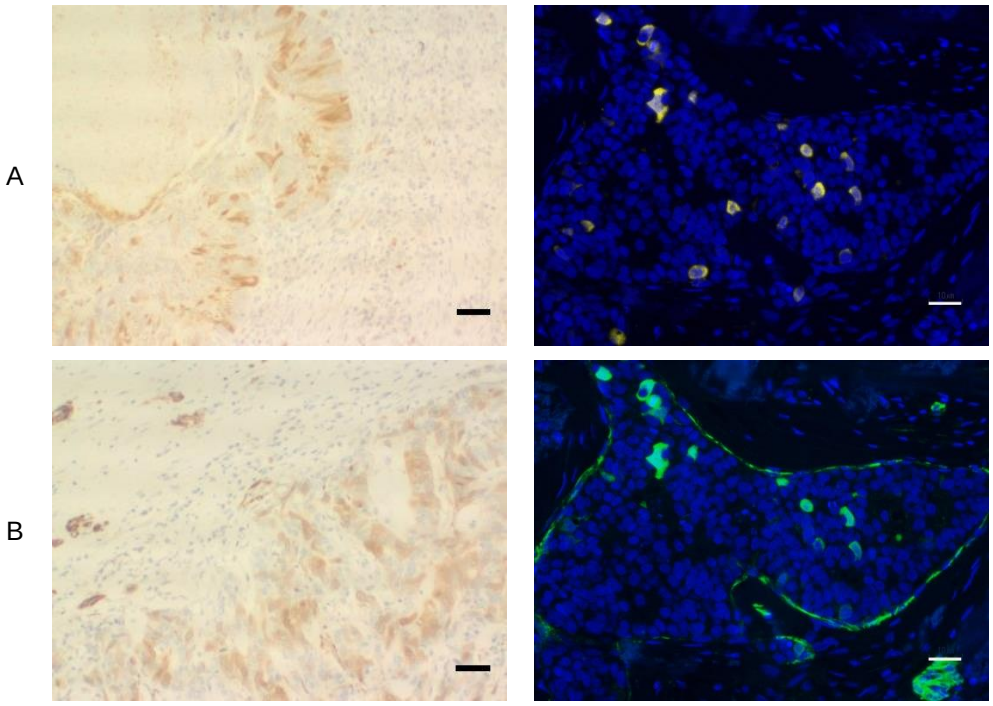


Figure 24. Comparison of expression patterns of securin in BC tissue shown by IHC and IF applying primary antibody clones DSC-280 and EPR 3240. Securin EPR 3240 demonstrated additional staining of collagen in basement membrane. Scale bar - 100 µm. A = DSC-280 and B = EPR 3240.

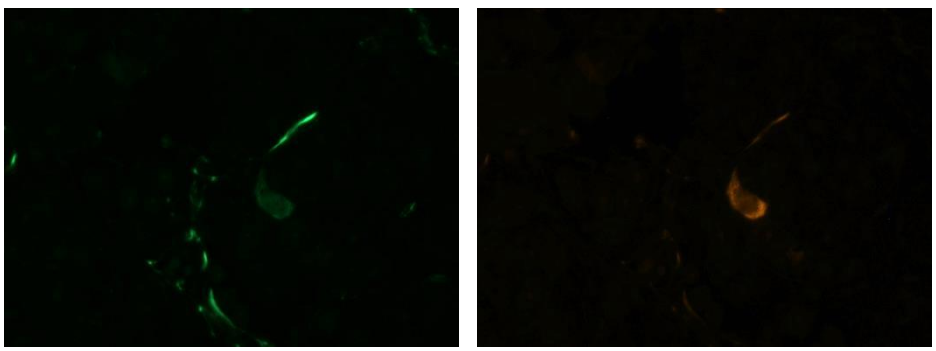


Figure 25. Double IF of Securin EPR 3240 (Alexa488, green) and Securin DSC-280 (Alexa555, yellow), x40 magnification.

4.3.2 PTTG1IP IHC and single IF

The expression pattern obtained by PTTG1IP primary antibody is demonstrated in figure 26. PTTG1IP showed different expression patterns in different types of breast cancer: in low grade carcinomas it was expressed abundantly, whereas in high grade carcinomas it was absent. IHC and IF provided identical staining pattern.

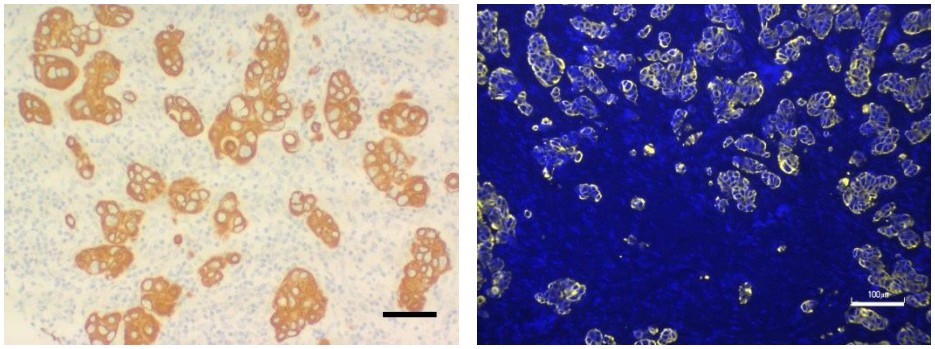


Figure 26. PTTG1IP is shown to be expressed in tumor epithelial cells in BC. Scale bar - 100 µm.

4.3.3 ki67 IHC and single IF

The expression pattern obtained by ki67 primary antibody is demonstrated in figure 27. IHC and IF provided identical staining pattern.

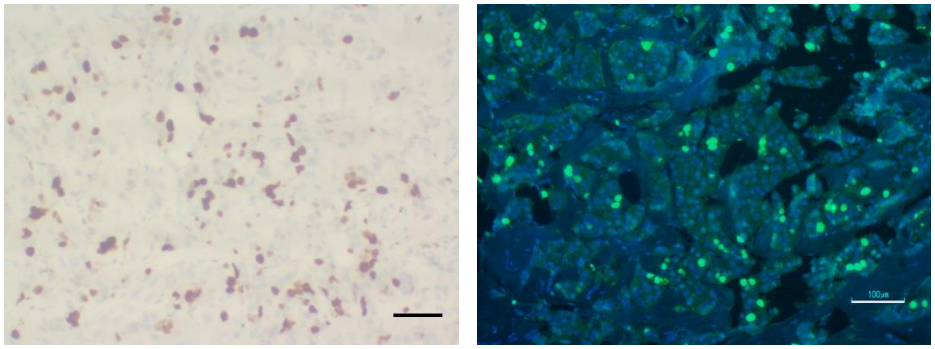


Figure 27. ki67 is shown to be highly expressed in breast cancer cells. Scale bar - 100 µm.

4.3.4 Double IF of selected biomarkers in BC

Expression patterns obtained by double IF for securin and PTTG1IP, as well as for securin and ki67 are shown in figures 28 and 29. In low grade breast carcinomas, securin-expression was seen in single cancer cells whereas Pttg1IP was observed diffusely in the majority of cancer cells. In triple-negative subtype of high grade breast carcinomas securin was highly expressed in the malignant cells but Pttg1IP expression was absent.

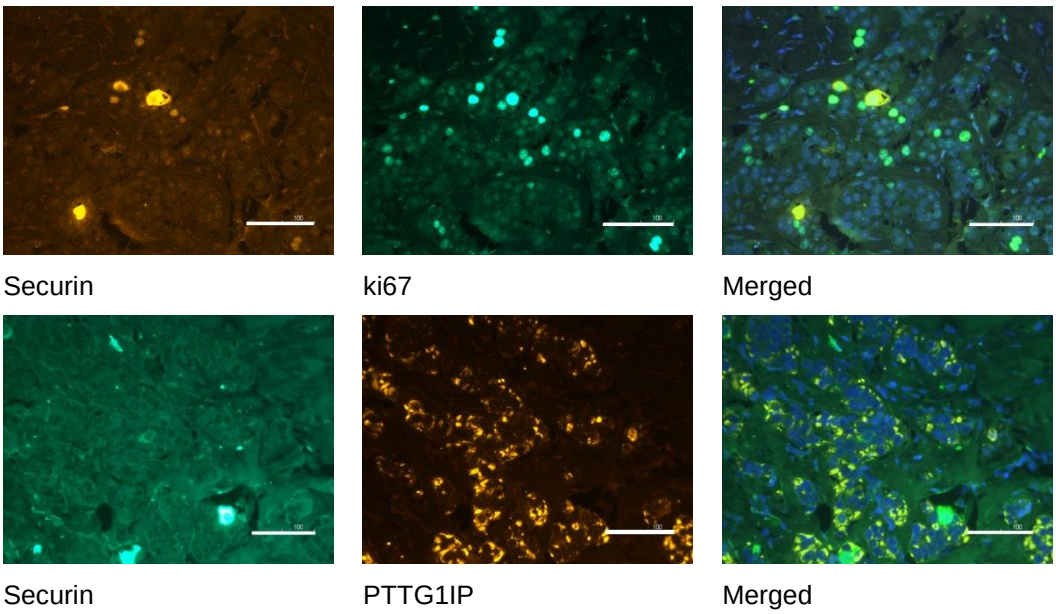


Figure 28. Double IF of selected biomarkers in low grade BC samples. 1. Securin DSC-280 and ki67. 2. Securin DSC-280 and PTTG1IP. In low grade breast carcinomas, securin-expression was seen in single cancer cells whereas Pttg1IP was observed diffusely in the majority of cancer cells. Scale bar - 100 µm.

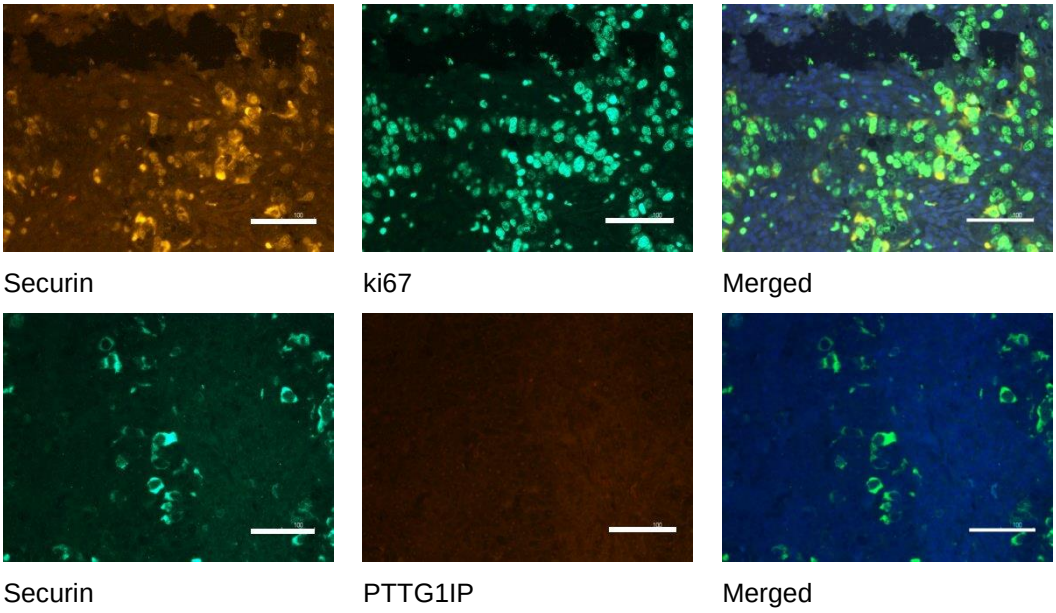


Figure 29. Double IF of selected biomarkers in triple-negative (high grade) BC samples. 1. Securin DSC-280 and ki67. 2. Securin DSC-280 and PTTG1IP. In triple-negative subtype of high grade breast carcinomas securin was highly expressed in the malignant cells but Pttg1IP expression was absent. Scale bar - 100 µm.

5. DISCUSSION

In this thesis, the main aim was to establish a technique for the visualization of multiple antigens in human formalin-fixed paraffin embedded tissue by immunofluorescence. It was shown that tyramide signal amplification system can be used to overcome the problem of autofluorescence seen in FFPE samples - intense signal from TSA exceeded that of biological structures. (Casella et al., 2004.) Notable exception was the autofluorescence from the connective tissue. This has been reported also previously (Abuduwali et al., 2013). Thus, care must be taken and appropriate control stainings must be included when using the developed protocol for the study of connective tissue. The experiments done in this study, however, focused mainly in the evaluation of biomarkers expressed in epithelial derived of tumor cells.

One problem of immunofluorescence technique is signal fading during storage (Mackie et al., 1980). Here we noticed, however, that signal intensity remains evaluable even after 1 year storage. This can be explained by storage in dark, stability and brightness of the utilized Alexa dyes, and also by sealing the edges of the coverslip with nail polish. Additionally, the applied mounting medium helps to secure the signal.

In this study, protocols were obtained for single fluorescent detection of ten biomarkers, including EGFR, p-mTOR, Ezrin, E-cadherin, Vimentin, Cytokeratin Pan, ki67, Cleaved caspase 3, securin and PTTG1IP. However, there were problems in the detection of pAkt, most probably, due to the delayed fixation of tissue. Two primary antibodies were tested for the detection of pAkt expression, but neither of them was able to show intense staining. In addition, small tissue biopsy gave better results compared to larger tissue. It is probable that small specimen has been fixed faster. Thus, it is more likely that problem is in tissue specimen, not in primary antibody. According to literature, phosphoproteins are especially prone to show alterations if fixation is delayed. Furthermore, stability of different phosphor-epitopes varies (Baker et al., 2005; Gündisch et al., 2011; Holzer et al., 2011).

In this thesis, sequential detection of two antigens was performed in double IF. In case of applying a pair of primary antibodies raised in different host species, there isn't problem of reaction between the first primary antibody and the second secondary antibody. However, it must be assured that the activity of the reporter enzyme used in the first detection is totally silenced before applying the second substrate on the slide. In the present study HRP was used as an reporter enzyme. Recently reported methods for destruction of the HRP activity after the first detection in double IF include

treatments using H_2O_2 (Roth K, 2008) and HCl (www.perkinelmer.com, 2015). In my experiments, these methods were not efficient enough. However, brief boiling in distilled water (Roth K, 2008), in this case 1 min, resulted in sufficient inactivation and was then successfully applied for further studies using both for colorectal and breast cancer tissues.

Double IF detection using primary antibodies raised in same host species can be more complicated. In addition to the enzyme inactivation process, also the antibodies already detected in the first sequence of the detection need to be removed, or their at least denatured. Universal commonly applicable method for the elution/denaturation is still not found, although numerous approaches have been introduced and their limitations studied in literature during the years. According to the data presented in this thesis, boiling in distilled water for 1 min was sufficient for the elimination of HRP activity, but even 3 min boiling was insufficient for the destruction of antibody itself. However, generalization of this result is limited by the number of tested antibodies. Nevertheless, ki67 – antibody which couldn't be removed by boiling – still showed promising results when harsher treatment including commercial acidic Riboclear-solution (ventana.com, 2014) combined with heat treatment was used. Utilization of microwave oven for the elimination of antibodies is known for decades (Lan et al., 1995), but the efficiency of this treatment remains still controversial (Bauer et al., 2001; Ikeda et al., 2011). In the experiments performed in this thesis, the method was not found efficient. But as with boiling, the result is limited by the number of tested antibodies. Elution using 2-ME/SDS is considered as a highly effective method for many antibodies, however, conditions required for successful elution have been shown to depend on the antibody affinity (Gendusa et al., 2014). In the current study, this method was considered rather efficient. The background fluorescence caused by SDS, however, disturbed the evaluation of the results. The idea to diminish fluorescence caused by SDS using Triton-X 100 was proposed by Lee et al. in (1978) for immunoelectroforetic analysis and was applied in this study for IF. The usage of Triton helped to decrease the background fluorescence, but it was not totally removed. Further experiments on this area could be beneficial since the results using the method were promising. According to Van der Loos (2008) heating at 98 °C for 10 min followed by cooling down to 75 °C is sufficient to remove some antibodies. In this study, this treatment was used in combination with acidic Riboclear-solution, and it proved to be efficient for several, but not for all, tested antibodies. The observed differences in the efficiency of elution/denaturation depend most likely on the individual characteristics, such as affinity (Gendusa et al., 2014), of the primary antibodies. Sometimes, the problem of cross reaction may be circumvented by applying the primary antibodies in different

order. For example, it was shown in this study that Cytokeratin Pan or Cleaved caspase 3 are recommended to be applied as late as in the second round of sequential double IF, whereas Vimentin, E-cadherin and ki67 can be applied in the first round since the treatment using Riboclear and heat was able to remove these antibodies.

As a summary, double IF was shown to be readily conducted when a pair of primary antibodies raised in different host species was applied. However, a universal procedure for a pair of primary antibodies raised in same host species was not found, although the combination of acidic solution Riboclear produced by Ventana and heating at 98 °C was shown to be suitable for some antibodies. In this thesis, only manual setting of fluorescent labelling was used but the gathered experiences can be readily adapted in future when transferring the stainings into the automated Discovery XT platform (Ventana). The tested combination of commercially available reagent Riboclear followed by heating can be easily performed on this platform. Automation of whole procedure from deparaffinization to fluorescent labelling of two antigens will help to resolve the problem of time-consuming and labor intensive optimization of double IF.

Multicolor immunofluorescence could be a beneficial tool for pathological diagnostics. However, the method is complicated. Present data indicated the possibility of simultaneous detection of two biomarkers in FFPE colorectal and breast cancer tissues shown by successful stainings applying many different primary antibody pairs raised in different host species (EGFR (clone 25)/p-mTOR, Ezrin/EGFR (clone 5B7), p-mTOR/Ezrin, Cytokeratin Pan/EGFR (clone 5B7), p-mTOR/Vimentin, p-mTOR/E-cadherin, Securin DSC-280/ki67 and Securin DSC280/PTTG1IP). Furthermore, double IF detection was also achieved in one pair of primary antibodies raised in same host species (Vimentin/Cytokeratin Pan). In this thesis, it has been shown that p-mTOR and EGFR were expressed in same tumor epithelial cells in CRC. Additionally, p-mTOR and Ezrin, p-mTOR and E-cadherin were co-expressed in cancer cells. However, p-mTOR was found in different tumor epithelial cells, and Vimentin was observed in mesenchymal cells. Same was true for EGFR and Cytokeratin Pan, as well as, for EGFR and Ezrin. In breast cancer, high proliferation shown by ki67 was associated with more numerous securin positive cells in tumor, although ki67 positivity was always found to exceed that of securin. In addition, low grade carcinomas were shown to express less securin compared to high grade cases. This was known already from literature (Talvinen et al., 2008). On the contrary, high expression of PTTG1IP was associated with low grade cancer, while high grade triple-negative subgroup showed absent expression of PTTG1IP (Gurvits et al., 2016).

6. CONCLUSIONS

Protocols for the visualization of many biomarkers in formalin-fixed paraffin embedded colorectal cancer and breast cancer samples using multicolor immunofluorescence method were developed and optimized. Detection of multiple markers in cancer tissues using these protocols could have an important role in translation to medical diagnostics. In this study, however, a limited number of antibodies, and especially a limited number of patient cases, were tested. To summarize the achieved results:

1. Single IF was successful, autofluorescence from connective tissue, however, must be controlled.
2. Double IF using a pair of primary antibodies raised in different host species was successful.
3. Double IF using a pair of primary antibodies raised in same host species was occasionally successful depending on the characteristics of the individual primary antibodies.
4. Application of multiple markers in the CRC cases was meaningful and provided information of the activation of signaling cascades in EGFR expressing cancer cells. In breast cancer, especially expression pattern of PTTG1IP was interesting, being highly expressed in low grade carcinomas, but absent in triple-negative carcinomas.

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9. APPENDICES

Table 7. Different combinations of conditions tested in the optimization of immunohistochemistry protocols

Antibody	Tissue	Antigen retrieval	Primary antibody dilution	Primary antibody diluent	Primary antibody incubation time, min	Secondary antibody incubation time, min
EGFR.25	CRC	sCC1	1:100	Ventana	36	20
		mCC2	1:100	Ventana	36	20
		mCC2	1:50	Ventana	36	20
		mCC2	1:50	Ventana	44	24
		mCC2	1:50	Ventana	52	24
		mCC2	1:50	Ventana	120	24
		mCC2	1:30	Ventana	52	24
		Manual	1:50	Ventana	52	24
pAKT EP209Y	CRC	mCC1	1:100	PSS	36	20
		mCC2	1:100	PSS	36	20
		sCC1	1:100	PSS	36	20
		mCC2	1:50	Ventana	32	12
		DAKO diluent 12 min, mCC2	1:50	Ventana	48	24
pAKT EP209Y	BC	mCC1	1:50	PSS	48	24
		mCC2	1:50	PSS	48	24
		sCC1	1:50	PSS	48	24
		Protease 3, 16 min	1:50	PSS	48	24
		Citrate buffer (pH 6.0) 32 min,sCC1	1:50	PSS	48	24
		DAKO diluent 12 min, mCC2	1:50	Ventana	48	24
		DAKO diluent 12 min, mCC2	1:50	DAKO	48	24
		DAKO diluent 12 min, mCC2	1:50	DAKO	60 RT	24
pAKT D9E	BC	mCC1	1:100	Ventana	36	20
		mCC2	1:100	Ventana	36	20
p-mTOR	CRC	sCC1	1:100	PSS	36	20

		sCC1	1:200	PSS	36	20
		sCC1	1:50	DAKO	36	20
		sCC1	1:100	DAKO	36	20
		sCC1	1:200	DAKO	36	20
		sCC1	1:50	Ventana	36	20
		sCC1	1:100	Ventana	36	20
		sCC1	1:200	Ventana	36	20
		sCC1	1:50	1% BSA in PSS	36	20
		sCC1	1:100	1% BSA in PSS	36	20
		sCC1	1:200	1% BSA in PSS	36	20
Ezrin 3C 12	CRC	mCC1	1:1000	Ventana	36	20
Securin rb	BC	mCC1	1:100	Ventana	36	20
		mCC1	1:200	Ventana	36	20
		mCC2	1:100	Ventana	36	20
		mCC2	1:200	Ventana	36	20
PTTG1IP	BC	mCC1	1:100	Ventana	36	20
		mCC1	1:200	Ventana	36	20
		mCC2	1:100	Ventana	36	20
		mCC2	1:200	Ventana	36	20

mCC1 - mild cell conditioning 1, mCC2 - mild cell conditioning 2, sCC1 - standard cell conditioning 1, sCC2 - standard cell conditioning 2.

Primary antibody incubation is done at 37 °C (except for those which are RT).

Table 8. Different combinations of conditions tested in single immunofluorescence protocols

Primary antibody	Tissue	Antigen retrieval	Dilution of primary antibody	Primary antibody diluent	Dilution of secondary antibody	Alexa	Dilution of Alexa	Signal
EGFR.25	CRC	6.0	1:25, 4 °C overnight	Ventana	1:50	Alexa488	1:50	Positive
	CRC	6.0	1:200, 4 °C overnight	Ventana	1:50	Alexa488	1:50	Negative
	CRC	6.0	1:50	Ventana	1:50	Alexa488	1:50	Negative
	CRC	6.0	1:50	Ventana	1:50	Alexa555	1:50	Negative
	CRC	6.0	1:50	Ventana	1:50	Alexa488	1:50	Negative
	CRC	6.0	1:25	Ventana	1:100	Alexa488	1:100	Negative
	CRC	6.0	1:25	Ventana	1:50	Alexa488	1:50	Negative
EGFR 5B7	CRC	9.0	200µl of antibody+50µl of diluent	Ventana	1:100	Alexa555	1:100	Positive
	CRC	6.0	200µl of antibody+50µl of diluent	Ventana	1:100	Alexa555	1:100	Positive
	CRC	9.0	200µl of antibody+50µl of diluent	Ventana	1:100	Alexa488	1:100	Positive
p-mTOR 2448 (49F9)	CRC	9.0	1:100	1% BSA/PSS	1:100	Alexa555	1:100	Positive

	CRC	6.0	1:100	1% BSA/PSS	1:100	Alexa488	1:100	Positive
Vimentin V9 790-2917	CRC	9.0	100µl of antibody+25µl of diluent	Ventana	1:100	Alexa488	1:100	Positive
	CRC	9.0	1:5	Ventana	1:100	Alexa488	1:100	Positive
	CRC	9.0	1:10	Ventana	1:100	Alexa488	1:100	Positive
	CRC	9.0	1:50	Ventana	1:100	Alexa488	1:100	Positive
	CRC	9.0	1:100	Ventana	1:100	Alexa488	1:100	Positive
	CRC	9.0	1:500	Ventana	1:100	Alexa488	1:100	Negative
E-cadherin NCH-38	CRC	9.0	1:100	Ventana	1:100	Alexa488	1:100	Positive
Cytokeratin Pan AE1/AE3	CRC	9.0	1:200	Ventana	1:100	Alexa555	1:100	Positive
	CRC	9.0	1:200	Ventana	1:100	Alexa488	1:100	Positive
pAKT 473 D9E	BC	9.0	1:100 4 °C overnight	Signal Stain (R)	1:100	Alexa555	1:100	Low
Ezrin 3C12	CRC	9.0	1:1000	Ventana	1:100	Alexa555	1:100	Positive
	CRC	9.0	1:1500	Ventana	1:100	Alexa488	1:100	Positive
	CRC	9.0	1:3000	Ventana	1:100	Alexa488	1:100	Positive
	CRC	9.0	1:10000	Ventana	1:100	Alexa488	1:100	Positive
	CRC	9.0	1:20000	Ventana	1:100	Alexa488	1:100	Positive
	CRC	9.0	1:50000	Ventana	1:100	Alexa488	1:100	Negative
Securin DSC-280	BC	6.0	1:150	Ventana	1:100	Alexa555	1:100	Positive
	BC	6.0	1:150	Ventana	1:100	Alexa488	1:100	Positive

	BC	9.0	1:150	Ventana	1:100	Alexa488	1:100	Positive
Securin EPR 3240	BC	6.0	1:1000	Ventana	1:100	Alexa555	1:100	Positive
	BC	6.0	1:1000	Ventana	1:100	Alexa488	1:100	Positive
	BC	9.0	1:100	Ventana	1:100	Alexa555	1:100	Positive
PTTG1IP	BC	6.0	1:1500	Ventana	1:100	Alexa555	1:100	Positive
Cleaved caspase 3 D3E9	LN	9.0	1:100	Ventana	1:100	Alexa488	1:100	Positive
ki67	LN	9.0	1:1000	Ventana	1:100	Alexa555	1:100	Positive
	LN	9.0	1:2000	Ventana	1:100	Alexa488	1:100	Positive

Primary antibody incubation is done at RT (except for those which are 4 °C).

Table 9. Optimized protocols for single immunofluorescence

Primary antibody	Tissue	Antigen retrieval	Dilution of primary antibody	Primary antibody diluent	Dilution of secondary antibody	Alexa	Dilution of Alexa
EGFR.25	CRC	6.0	1:25, 4 °C overnight	Ventana	1:50	Alexa488	1:50
EGFR 5B7	CRC	6.0	200µl of antibody+50µl of diluent	Ventana	1:100	Alexa555	1:100
	CRC	9.0	200µl of antibody+50µl of diluent	Ventana	1:100	Alexa488	1:100
p-mTOR 2448 (49F9)	CRC	9.0	1:100	1% BSA/PSS	1:100	Alexa555	1:100

	CRC	6.0	1:100	1% BSA/PSS	1:100	Alexa488	1:100
Vimentin V9 790-2917	CRC	9.0	1:100	Ventana	1:100	Alexa488	1:100
E-cadherin NCH-38	CRC	9.0	1:100	Ventana	1:100	Alexa488	1:100
Cytokeratin Pan AE1/AE3,	CRC	9.0	1:200	Ventana	1:100	Alexa555	1:100
	CRC	9.0	1:200	Ventana	1:100	Alexa488	1:100
Ezrin 3C12	CRC	9.0	1:1000	Ventana	1:100	Alexa555	1:100
	CRC	9.0	1:20000	Ventana	1:100	Alexa488	1:100
Securin DSC-280	BC	6.0	1:150	Ventana	1:100	Alexa555	1:100
	BC	6.0	1:150	Ventana	1:100	Alexa488	1:100
Securin EPR 3240	BC	6.0	1:1000	Ventana	1:100	Alexa555	1:100
	BC	6.0	1:1000	Ventana	1:100	Alexa488	1:100
PTTG1IP	BC	6.0	1:1500	Ventana	1:100	Alexa555	1:100
Cleaved caspase 3 D3E9	LN	9.0	1:100	Ventana	1:100	Alexa488	1:100
ki67	LN	9.0	1:1000	Ventana	1:100	Alexa555	1:100

Primary antibody incubation is done at RT (except for those which are 4 °C).

Table 10. Experiments with different methods of destruction in double IF protocol with primary antibodies raised in the same species

Method	First primary antibody	First Alexa	Second primary antibody	Second Alexa	Efficacy of destruction
1 min boiling	ki67	Alexa555	Cleaved caspase 3	Alexa488	low
1 min boiling	ki67	Alexa555	Antibody diluent	Alexa488	low
3 min boiling	ki67	Alexa555	Cleaved caspase 3	Alexa488	low
3 min boiling	ki67	Alexa555	Antibody diluent	Alexa488	low

Microwave treatment	ki67	Alexa555	Cleaved caspase 3	Alexa488	low
Riboclear	ki67	Alexa555	Cleaved caspase 3	Alexa488	low
Riboclear and short boiling	ki67	Alexa555	Cleaved caspase 3	Alexa488	low
Riboclear and H_2O_2	ki67	Alexa555	Cleaved caspase 3	Alexa488	low
Riboclear and heating 98 °C	ki67	Alexa555	Cleaved caspase 3	Alexa488	promising
Riboclear and heating 98 °C	Cytokeratin	Alexa555	Vimentin	Alexa488	low
Riboclear and heating 98 °C	Vimentin	Alexa488	Cytokeratin	Alexa555	high
Riboclear and heating 98 °C	Antibody diluent	Alexa555	Vimentin	Alexa488	high
Riboclear and heating 98 °C	Cytokeratin	Alexa555	Antibody diluent	Alexa488	low
Riboclear and heating 98 °C	Vimentin	Alexa555	Cytokeratin	Alexa488	high
Riboclear and heating 98 °C	Vimentin	Alexa488	Antibody diluent	Alexa555	high
Riboclear and heating 98 °C	Cytokeratin	Alexa488	Vimentin	Alexa555	low
Riboclear and heating 98 °C	Cytokeratin	Alexa488	Antibody diluent	Alexa555	low
Riboclear and heating 98 °C	Cleaved caspase 3	Alexa555	Antibody diluent	Alexa488	low
Riboclear and heating 98 °C	E-cadherin	Alexa488	Antibody diluent	Alexa555	high
ME/SDS 56C and PBS washing	ki67	Alexa555	Cleaved caspase 3	Alexa488	green autofluorescence from SDS
ME/SDS 56C and PBS and Triton X-100 washing	ki67	Alexa555	Cleaved caspase 3	Alexa488	promising
ME/SDS 56C and PBS and Triton X-100 washing	Cleaved caspase 3	Alexa555	Antibody diluent	Alexa488	green autofluorescence from SDS

Table 11. Application of different antibodies in the best denaturation method Riboclear and heating 98 °C

First primary antibody	Type of antibody	First Alexa	Second primary antibody	Type of antibody	Second Alexa	Efficacy of destruction
ki67	rabbit polyclonal	Alexa555	Cleaved caspase 3	rabbit monoclonal	Alexa488	promising
Cytokeratin Pan	mouse monoclonal	Alexa555	Vimentin	mouse monoclonal	Alexa488	low
Vimentin	mouse monoclonal	Alexa488	Cytokeratin Pan	mouse monoclonal	Alexa555	high
Antibody diluent	-	Alexa555	Vimentin	mouse monoclonal	Alexa488	high
Cytokeratin Pan	mouse monoclonal	Alexa555	Antibody diluent	-	Alexa488	low
Vimentin	mouse monoclonal	Alexa555	Cytokeratin Pan	mouse monoclonal	Alexa488	high
Vimentin	mouse monoclonal	Alexa488	Antibody diluent	mouse monoclonal	Alexa555	high
Cytokeratin Pan	mouse monoclonal	Alexa488	Vimentin	mouse monoclonal	Alexa555	low
Cytokeratin Pan	mouse monoclonal	Alexa488	Antibody diluent	-	Alexa555	low
Cleaved caspase 3	rabbit monoclonal	Alexa555	Antibody diluent	-	Alexa488	low
E-cadherin	mouse monoclonal	Alexa488	Antibody diluent	-	Alexa555	high

Appendix 1. Calculations for 2% H_2O_2

The volume of was calculated for 75 ml container:

$$\frac{V_1}{V_2} = \frac{c_2}{c_1}$$

$$V_1 = \frac{V_2 \times c_2}{c_1}$$

$$x = \frac{75\ ml\ \times\ 2\%}{30\ \%}$$

$$x = 5\ ml\ of\ 30\% \quad H_2\ O_2$$

Appendix 2. Calculations for 0.01 M HCl

The volume of HCl was calculated as the following:

$c = 0.01\ M\ HCl,\ \omega = 37\%\ HCl,\ \rho = 1{,}184 \frac{g}{cm^3},\ m = 1{,}189\ g,\ V = 1l,\ MW = 36.59\ g/mol$

$$\rho = \frac{m}{V}$$

$$\omega = \frac{m_{dissolved\ substance}}{m_{solution}}$$

$$m_{dissolved\ sustance} = \omega \times m_{solution}$$

$$m_{dissolved\ sustance} = 0.37 \times 1{,}184 \frac{g}{cm^3} = 0.44\ kg = 440g$$

$$c = m_{dissolved\ substance} \div MW \div V$$

$$c = 440g \div 36.59 \frac{g}{mol} \div 1l = 12.06\ M$$

$$\frac{V_1}{V_2} = \frac{c_2}{c_1}$$

$$V_1 = \frac{V_2 \times c_2}{c_1}$$

$$V_1 = \frac{0.1l \times 0.01\ M}{12.06\ M} = 0.00008l = 8ml\ of\ 37\%\ HCl\ in\ 100\ ml\ MQ - H_2O$$

Appendix 3. Calculation for 3% H_2O_2

The volume of H_2O_2 was calculated for 75 ml container:

$$\frac{V_1}{V_2} = \frac{c_2}{c_1}$$

$$V_1 = \frac{V_2 \times c_2}{c_1}$$

$$x = \frac{75\ ml\ \times\ 3\%}{30\ \%}$$

$$x = 7{,}5\ ml\ of\ 30\% \quad H_2\ O_2$$

Appendix 4. Calculations for Tris-HCl buffer pH 6.8

The weight of dry Trisma base for Tris-HCl 0.5 M, 0.5l was calculated as following:

$$n = \frac{m}{M}\ ;\ m = n \times M$$

$$c = \frac{n}{V}\ ; n = c \times V$$

Substituting n from the second formula to the first was calculated the required weight of Trisma base:

$$m = c \times V \times M$$

$$m = 0.5\ M \times 0.5l \times 121{,}14\frac{g}{mol}\ ;\ m = 30.1\ g$$

The buffer was calibrated to pH 6.8 using HCl.

Appendix 5. Prerapation of peroxidase quenching buffer

10x PBS - 7,5 ml; distilled water - 60 ml; 30% H_2O_2 - 7,5 ml.

Appendix 6. Prerapation of BSA.

1x PBS - 5ml; BSA - 0,05 g.

Appendix 7. Preparation of 10x PBS.

80g NaCl , 2g KCl 144g, Na2HPO4 and 2,4g KH2PO4). pH was adjusted to 7.4 using 5M sodium hydroxide.

Appendix 8. Preparation of 2-ME/SDS solution

20 ml of 10% w/v SDS (10g of SDS in 100ml of distilled water), 12,5ml of 0.5 M Tris-HCl, 67.5ml of distilled water and 0.8 ml of 2-ME.