Assay development for *in situ* detection of autophagy-related protein-protein interactions for characterization of colorectal cancer

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Abbreviations

Atg  
Autophagy Related
AMP  
Adenosine Monophosphate
ATP  
Adenosine Triphosphate
Bcl-2  
B-cell lymphoma 2
BNIP3  
Bcl-2/adenovirus E1B 19-kDa Interacting Protein 3
cDNA  
Complementary Deoxyribonucleic Acid
CRC  
Colorectal Cancer
DMEM  
Dulbecco's Modified Eagle Medium
DNA  
Deoxyribonucleic Acid
dNTP  
Deoxynucleotide
Endo IV  
Endonuclease IV
ER  
Endoplasmic Reticulum
EtOH  
Ethanol
FBS  
Fetal Bovine Serum
HIF-1  
Hypoxia Inducible Factor-1
HPLC  
High-Performance Liquid Chromatography
isPLA  
In situ Proximity Ligation Assay
isRCA  
In situ Immuno-Rolling Circle Amplification
LC3  
Microtubule-associated Protein 1A/1B-Light Chain 3
MEM  
Minimum Essential Medium
mRNA  
Messenger Ribonucleic Acid
mTOR  
Mammalian Target of Rapamycin
mTORC1  
Mammalian Target of Rapamycin Complex 1
NHS  
N-hydroxysuccinimide
PBS  
Phosphate Buffered Saline
PCR  
Polymerase Chain Reaction
PE  
Phosphatidylethanolamine
PEST  
Penicillin/Streptomycin Solution
PFA  
Paraformaldehyde
PI3  
Phosphoinositide 3-kinase
PI3P  
Phoshatidyl Inositol Triphosphate
PNK  
Polynucleotide Kinase
RCA  
Rolling Circle Amplification
RCR  
Rolling Circle Product
RNA  
Ribonucleic Acid
SDS-PAGE  
Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SANH  
Succinimidyl 6-hydrazinonicotinate Acetone Hydrazone
SMCC  
Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate
SQSTM1  
Sequestosome-1
SSC  
Saline-Sodium Citrate
TBST  
Tris-Buffered Saline with Tween-20
TCEP  
Tris(2-carboxyethyl)phosphine
TEM  
Transmission Electron Microscopy
ULK  
Unc-51-Like Kinase
UNG  
Urasil DNA Glycosylase
Vps  
Vesicular Protein Sorting
Abstract

Every year, more than a million people are diagnosed with colorectal cancer (CRC) that develops in the large intestine. It is one of the most studied cancers in the world but still more knowledge about how this cancer develops and acts is needed in order to use more effective ways to treat CRC. Autophagy is a vital mechanism in cells that is also suggested to maintain cancer cell survival. In normal cells, it plays an important role by removing damaged cells and organelles as well as eliminating pathogens. Under metabolic stress this mechanism is induced to provide enough nutrients and energy for the cell to survive. Cancer cells are exposed to greater environmental stress than normal cells and therefore, cancer cells exhibit higher levels of autophagy suggesting it to be a crucial mechanism for their survival. Gaining a deeper understanding of this essential mechanism and its activation might provide new insights and improved treatments for the fight against colorectal cancer. *In situ* Proximity Ligation Assay (PLA) is a protein detection method that enables sensitive and specific detection of proteins and protein-protein interactions (PPIs) in cell lines and tissue samples. The method uses simultaneous recognition of two independent antigens on a protein or protein complex together with a rolling circle amplification (RCA) to form a rolling circle product (RCP) on top of the target. By using fluorescent oligonucleotides, RCP can be visualized and is seen as a bright spot that enables sensitive detection of the target at single-molecule resolution. The aim of this study was to develop assays to detect endogenous molecular events known to be biomarkers of autophagy *in situ* in order to study autophagy mechanism in CRC patient samples. We focused our research on two PPIs that were known to interact when autophagy is induced. The first investigated interaction was between microtubule-associated protein 1A/1B-light chain 3 (LC3) and sequestome-1 (SQSTM1), an interaction that occurs during autophagy initiation. The second interaction was between B-cell lymphoma 2 (Bcl-2) and Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) that takes place during hypoxia-induced autophagy. To study whether these PPIs can be used as a detection method to monitor autophagy, we used a well-established cell model based on serum starvation and CoCl$_2$ - an hypoxic mimetic- treatment of the intestinal cancer cell line Caco-2 in comparison to normal culture condition. According to *isPLA* quantification, detection of both PPIs was distinctly higher in treated cells compared to untreated cells giving promising results and suggesting that they can be potentially used as suitable assays to monitor these biomarkers of autophagy. For development of an improved protein detection method that enables the study of several PPIs simultaneously in a tissue sample (*In situ* Multiplexing), we conjugated directly a short oligonucleotide strand to the primary antibodies. These formed proximity probes could later be used in *in situ* for multiplexing.
**Introduction**

**Biology**

**Colorectal cancer**

The digestive system is composed of all organs in the body that are involved in digestion of food to provide nutrients and energy for the cells. The large intestine is a 1.5 m long part of the digestive system. In this report we focus on better characterization of colorectal cancer (CRC), a cancer that develops in the large intestine. The main role of the large intestine is to absorb remaining water and nutrients from partly digested food and to form a solid or semisolid fecal material. Large intestine can be divided into four major parts that are cecum, colon, rectum and anal canal. The wall of the large intestine has four layers in which the mucosa forms the closest layer to lumen (Tortora and Derrickson, 2009). The mucosa has a monolayer of epithelium and intestinal glands that forms tubular “crypts” extending through the mucosa. Differentiated cells are located in the top third of the crypts and are derived from the stem cells residing at the bottom (Sancho et al., 2004).

CRC is the third most commonly diagnosed cancer in the USA also being the third leading cause of cancer deaths (American Cancer Society, 2014). CRC develops slowly. In most cases, it begins with noncancerous growths on the epithelium called polyps. They normally do not cause any symptoms but may develop cancerous over time (Tortora and Derrickson, 2009). These tumors of epithelial with glandular origin or adenocarcinomas develop from mutated epithelial stem cells. Originally it was believed that any normal cell could become cancerous over time. However, latest studies have suggested that CRC develops from long-lived stem cells since intestinal epithelial cells does not exist long enough to develop tumor inducing changes (Todaro et al. 2010). An early diagnosis of CRC shows good prognosis. However, in most cases the cancer develops to a later stage associated to a poor rate of survival. To treat the cancer, surgical removal of the tumor is used in most cases. In order to decreases CRC-related mortality, intensive screening is mandatory but it is equally important to understand how CRC cancer develops and acts allowing the discovery of more effective treatments (Tortora and Derrickson, 2009).

**Autophagy**

Autophagy is a crucial cellular mechanism that is initiated under metabolic stress in order to promote cell survival both in normal and cancer cells. Autophagy is a conserved, self-degradative molecular pathway in Eukaryotic cells. It plays a vital housekeeping role in cells where it removes misfolded, aggregated or obsolete proteins, clears damaged organelles such as mitochondria and endoplasmic reticulum (ER) as well as eliminates intracellular pathogens (Glick et al., 2010; Xie and Klionsky, 2007). Under metabolic stress, autophagy works as an adaptive response to provide enough nutrients and energy for the cell. The mechanism has implications to various fields such as development, aging and cancer (Yang and Klionsky, 2010). There are three forms of autophagy present in the cell: macroautophagy, microautophagy and chaperone-mediated autophagy (Glick et al., 2010). However, in this work we focus on macroautophagy that is hereafter referred as ‘autophagy’.
Autophagy in cancer

Within the past ten years, many studies have highlighted the link between autophagy and cancer progression. In cancer cells the inherent stress is greater than in normal cells due to a faster cell proliferation rate, increased metabolic demand and poor blood supply due to a deficient vascularization. Therefore, tumor cells exhibit higher levels of autophagy than normal cells (White and DiPaola, 2009). Even though it is still controversial, autophagy is considered to assist cancer cell survival. Though, other hypotheses suggest that it can also inhibit cancer cell survival. Once autophagy is pushed beyond a certain point, it triggers a cell death mechanism such as apoptosis to eliminate cells that are damaged or dangerous. Nevertheless, tumor cells frequently develop ways to disrupt genes involved in apoptosis and cell proliferation in order to survive (Lei et al., 2014, White and DiPaola, 2009). A better understanding of the autophagic pathway is urgent since it might provide new insights to treatment and prevention of cancer and thus, methods to analyze it are needed.

Autophagy machinery

The autophagic machinery begins with the isolation of a double-membrane phagophore that engulfs cytoplasmic cargo forming a vesicle called autophagosome. Until now, it has been controversial where the phagophore membrane originates but according to an emerging consensus it originates from the subdomain of ER (Biazik et al., 2015). After fusion of the autophagophore with the lysosome, the lysosomal acid proteases degrade the loaded autophagosome (Figure 1). With the help of the lysosomal permeases and transporters, amino acids and other by-products are transferred back to the cytoplasm. There, they can be metabolized or alternatively reused to build macromolecules (Glick et al., 2010). The phagophore formation is initiated by the ULK complex and the vesicular protein sorting 34 (Vps 34) and Beclin 1 complex (Glick et al., 2010; Petiot et al., 2000; Kihara et al., 2001). Under nutrient-enriched conditions, mammalian target of rapamycin (mTOR) complex 1 (mTORC1) inactivates two essential components, unc-51-like kinase (ULK) and Atg13 by phosphorylating them. Under nutrient-depleted conditions, mTORC1 activity is inhibited and thus autophagy is induced by ULK complex (Mathew et al., 2011). ULK complex recruits Vps34 to the phagophore (Russell et al., 2014). Several proteins can interact with the Vps34-Beclin 1 complex either to promote or inhibit autophagy. Among others, B-cell lymphoma 2 (Bcl-2) acts as an autophagy inhibitor when bound to Beclin 1 (Glick et al., 2010, Pattingre et al., 2005). When the phagophore elongates more proteins are recruited. Autophagy has been initially studied in yeast resulting in the discovery of the autophagy-related (Atg) genes, which are highly conserved among mammals and plants (Nakatogawa et al., 2009). The multimeric Atg5-Atg12-Atg16L complex associates with the growing phagophore and induces its curvature by recruiting the microtubule-associated protein 1A/1B-light chain 3 (LC3) also known as Atg8 in yeast. Even though conjugated Atg5-Atg12 is tightly involved in the phagophore formation, it cannot be used as a biomarker for autophagy since it dissociates from the phagophore membrane. LC3 is a microtubule-associated protein that is expressed in almost all cell types. Upon induction of autophagy, LC3 is proteolytically cleaved by Atg4 to expose the C-terminal glycine, now called as LC3-I. LC3-I is subsequently activated by Atg7 and transferred to Atg3 where a phosphatidylethanolamine (PE) is conjugated to the glycine group of LC3-I to form LC3-II. The conjugated PE group then contributes to the integration of LC3-II into the phagophore membrane. LC3-II is located to the internal and external surface of the phagophore where it selects cargo for the degradation and participates in the hemifusion of the membranes. LC3-II can be used as an autophagic biomarker since its synthesis and processing is consistently increased during autophagy and it does not dissociate from the phagophore membrane (Glick et al., 2010). However, once autophagosome fuses with lysosome LC3-II proteins in the inner membrane are degraded whereas...
the ones on the outer membrane will be deconjugated and returned to cytoplasm (Mizushima and Yoshimori, 2007). In this work bafilomycin A₁ treatment was used since it prevents the fusion between autophagosomes and lysosomes thus accumulating autophagosomes (Yamamoto et al., 1998). The most well-known protein interacting with LC3-II is the ubiquiting-binding protein sequestosome-1 (SQSTM1) also known as p62. SQSTM1 promotes the uptake of polyubiquitinated protein aggregates thus facilitating the autophagic flux (Hale et al., 2013).

Hypoxia-induced autophagy

The reduction of oxygen supply below the physiological level, for example within a tumor, is called hypoxia (Nikinmaa, 2013). Under hypoxic stress, the autophagy of mitochondria or mitophagy is triggered in order to promote cell survival. Hypoxia-induced autophagy is controlled by the transcription factor hypoxia inducible factor-1 (HIF-1) that is a common regulator of the oxygen homeostasis (Zhang et al., 2008). In response to hypoxia, HIF-1 regulates the transcription of hundreds of genes among which, the pro-autophagic protein Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3). BNIP3 induces mitochondrial autophagy by disrupting the interaction between Beclin 1 and Bcl-2. BNIP3 dissociates Beclin 1-Bcl-2 complex by using its BH3 domain and thus displaces Beclin 1 from the complex and binds to Bcl-2. It is suggested that BNIP3 acts independently of mTOR in order to dissociate Beclin 1-Bcl-2 complex (Bellot et al., 2009).
Current detection of autophagy

The importance of the autophagic machinery in diseases such as CRC has pushed scientists to develop reliable assays to monitor this mechanism in situ in cell lines or tissue samples. Currently, there are few detection methods available. Autophagy has been initially characterized by transmission electron microscopy (TEM), a method that is still commonly used. Even though the method allows sensitive detection of autophagic vesicles, it has received criticisms for being not objective enough since the interpretation of the TEM pictures is based on the identification of the characteristic double-membrane and depends on how skilled the person is (Eskelinen, 2008). The demand of more quantitative methods drives the investigation for alternative options. One of the most investigated proteins to monitor autophagy is LC3-II for which the expression is known to increase during autophagy. A decreased amount of LC3-I and an increased amount of LC3-II detected by SDS-PAGE and immunoblotting indicates that autophagy is induced in cells. However, commercially available antibodies directed against LC3 detect the LC3-II form more sensitively than LC3-I form and thus, the absolute autophagy activity cannot be measured reliably (Mizushima and Yoshimori, 2007). Fluorescent microscopy has been an alternative method to monitor autophagy in situ. Use of green fluorescent protein (GFP) fused to LC3 is used widely but several drawbacks have been detected including the GFP sensitivity to lysosomal proteases and the limitation to transfectable cell lines thus excluding patient samples (Barth et al., 2010; Kimura et al., 2007). Standard immunohistochemical methods can be also used to detect LC3-II levels as well as other autophagy related proteins whose expression is increased during autophagy induction such as SQSTM1 and Beclin 1. According to some studies the use of immunohistochemical methods is not preferred due to doubts about the specificity as mentioned before. For example, it has been shown that during decreased autophagic activity, some proteins may accumulate in the cytoplasm (Martinet et al., 2012). Finally, most of the studies on cell lines are using chemical inhibitors of the autophagic degradation such as bafilomycin inducing an accumulation of the autophagic vesicles and thus facilitating their detection. In order to study patient material, is it important to be able to monitor endogenous level of autophagy. There is still a need for more specific and trustworthy methods to be developed.

Methods

All proteins physically interact with other molecules. The interaction with other molecules is significant since it determines the biological properties of a protein (Alberts et al., 2002). In order to study dynamic cellular processes, such as autophagy, in the living cells or tissue sections, sensitive, specific and reliable methods are required.

In situ single protein and PPIs detection Assay

In 2002 a study of Fedrikson et al. demonstrated a method termed proximity ligation, which showed how proteins are sensitively and specifically detected in vitro by using two affinity probes to which single stranded oligonucleotides are conjugated. If these two proximity probes bind epitopes close enough to each other, subsequently added connector oligonucleotides hybridize to the conjugated oligonucleotides. Once the remaining nick between these two oligonucleotides is ligated, the ligation product could serve as a template for polymerase chain reaction (PCR). Already when this method was first published, an idea of applying it in situ for protein detection was suggested (Fredrikson et al., 2002). In a similar way, in situ Proximity Ligation Assay (isPLA) utilizes
simultaneous dual epitopes recognition to detect single proteins, protein-protein interactions and post-translational modifications at single-molecule resolution in cell lines or tissue samples.

**Detection of protein expression in situ by Proximity Ligation Assay**

*IsPLA* (Figure 2) utilizes dual recognition to detect proteins, protein-protein interactions and post-translational modifications in single-molecule resolution in cell lines or tissue samples. Due to the dual recognition, the specificity compared to conventional immunohistochemical staining methods is increased. First, two affinity reagents, most commonly antibodies, bind in a close proximity to two different epitopes on a target (Söderberg *et al.*, 2006; Söderberg *et al.*, 2008). There are several affinity reagents currently on the market used for studying proteins and PPIs. However, in this work antibodies were used as affinity reagents. Proximity probes can either be primary antibodies or secondary antibodies depending on the assay used. Only when the two proximity probes are bound close enough to each other on a protein or protein complex, the oligonucleotide strands form a bridge between the two antibodies. Right after connector oligonucleotides, so called “splint” and “backpiece” are added. Both of these oligonucleotides have sequences that can hybridize to the proximity probes. With a help of a DNA ligase, the remaining nicks are sealed to form a circular DNA molecule. The circular DNA molecule can thus serve as a template for rolling circle amplification (RCA). Addition of highly processive phi29 DNA polymerase starts the synthesis of a single-stranded DNA molecule (Söderberg *et al.*, 2006). One of the proximity probes serves as a primer probe and remains attached the rolling circle product (RCP), while the other probe has three mismatched RNA nucleotides at the end, which prevent the priming. The ability of phi29 to synthesize DNA chains without dissociating it from the template makes it ideal to use in *isPLA* assay since it keeps the subcellular localization of the molecule (Blanco *et al.*, 1989; Söderberg *et al.*, 2006). A formed single-stranded RCP on top of the target can be visualized with fluorescence-labeled detection oligonucleotides. The circular DNA template includes an encoded sequence that is complementary to a detection oligonucleotide. Since there are hundreds of copies of the DNA circle, there are hundreds of detection oligonucleotides bound to the RCP. This is seen as a bright spot that is easily detectable with regular fluorescence microscopy (Söderberg *et al.*, 2006). The quantification of the signal is possible by using cell image analysis software such as CellProfiler (www.cellprofiler.org) (Carpenter *et al.*, 2006).

**Detection of protein expression in situ by Immuno-Rolling Circle Amplification**

*In situ* Immuno-rolling circle amplification (*isRCA*) is a similar method to *isPLA*. Unlike *isPLA*, *isRCA* is not based on a dual recognition for protein detection. Instead, the assay uses a single probe that binds to the target molecule and then takes advantage of the distinct RCP. This is made possible by hybridizing a padlock probe to the oligonucleotide conjugated to the proximity probe. The padlock probe has two hybridizing regions: one at the 5’end of the probe and one at the 3’end that are both complementary to adjacent regions on the oligonucleotide strand attached to the antibody. Therefore, the padlock can form a circle after ligation of the nick enabling RCA by the phi29 polymerase. The RCP can be visualized by adding the detection oligonucleotides that will hybridize to the RCP as described previously and form a bright that in visualized by fluorescence microscopy (Nilsson *et al.*, 1994; Larsson *et al.*, 2004).
Due to the dual recognition, the specificity is increased compared to conventional immunohistochemical staining methods but the affinity reagents efficiency and specificity remains a key feature of the assay. In this work antibodies were used as affinity binders but there are also other affinity binders that specifically bind to targets such as aptamers that are small nucleic acid molecules, designed ankyrin repeated proteins (DARPins) that have a variable and modular binding surface and nanobodies that are small single domain antibodies (Ni et al., 2012; Binz et al., 2003; Cortez-Retamozo et al., 2004).

**Affinity Probes**

Antibodies are molecules that defend the body against infection by attacking pathogens. There are billions of forms of antibodies, each carrying a unique antigen-binding site. This ability to bind specifically on an epitope on the target makes them desirable to use as affinity binders. Each tip of the two “arms” has identical antigen-binding sites that involve both the light and the heavy chain. The antigen binding sites are located in the variable domains. The molecule is held together by a set of noncovalent bonds and covalent disulfide bonds (Alberts et al., 2002). Each antibody contain both intra-chain and inter-chain disulfide bonds. To hold the molecule together, the two heavy chains are connected in the hinge region by a variable number of disulfide bonds and both light chains are connected to heavy chains by one disulfide bond (Liu and May, 2012). When an antibody binds to an antigen several weak, non-covalent forces are formed to hold the molecules together. The binding is always reversible and its strength depends on the number of binding site as well as the affinity, which explain the variation of efficiency of probes (Alberts et al., 2002).
Sulfo-Succinimidyl 6-hydrizinonicinate acetone hydrazine (SANH) conjugation (Sulfo-SANH)

Ideally, a probe contains one affinity molecule that is linked, usually through a covalent bond, to an oligonucleotide (Hermanson 2008). There are several methods to form proximity probes but in this report the focus is on sulfo-SANH method and sulfo-SMCC method respectively. The most notable difference between the two conjugation methods is that SANH molecule can bind to the N-terminal end of the molecule and thus affect the affinity on the antigen-binding site whereas SMCC binds to the sulfhydryl groups and thus does not modify the binding affinity and specificity. We would like to optimize the reverse-SMCC conjugation method for further use in antibody conjugation but since it is still under optimization we are interested in investigate the efficiency and specificity of both methods.

SANH is a commonly used heterobifuctional conjugation reagent (Figure 3) that has an amine reactive N-hydroxysuccinimide (NHS-ester) on one end of the molecule and a hydrazine group on the other end. Unlike SANH, sulfo-SANH has a negatively charged sulfonate group on the NHS ring, which provides sufficient polarity to the molecule to dissolve in water. NHS-ester couples with primary amines at the N-terminal end of the molecule and thus forms a stable amide bond. The hydrazine group form hydrazone bonds with carbonyl compounds such with aldehydes and ketones (Hermanson 2008). SANH is commonly used for antibody-DNA conjugation. The NHS-ester directly reacts with the free amines on the antibody at physiological or slightly basic pH. After removing the excess SANH, the hydrazine group of the SANH-modified antibody can reach the aldehyde modified-oligonucleotide forming a covalent hydrazine bond (Koslov et al., 2004).

The picture is not available in the electronic version for reasons of copyright

Figure 3. SANH-conjugation. The NHS-ester of SANH reagent binds to primary amine group of a protein. Once the SANH modified protein is mixed with aldehyde-modified oligonucleotides the hydrazine group forms hydrazone bonds with the aldehyde group forming and protein-oligonucleotide conjugate. (Picture: Wu et al., 2010)
**Sulfo-Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) conjugation (Sulfo-SMCC)**

SMCC is another reagent that can be used for conjugation of two molecules. Like sulfo-SANH, sulfo-SMCC is a biofunctional and water-soluble crosslinker that contains NHS-ester and a sulfhydryl reactive maleic acid imide (maleimide) group (Figure 4). The maleimide group on the other end of the molecule undergoes an alkylation reaction with sulfhydryl group and thus can form a stable thioether bond. The reaction between SMCC and sulfhydryl is specific at pH 6.5-7.5. (Hermanson 2008).

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) is a solid, water soluble and odorless trivalent phosphine known to reduce disulfide bonds (Burns et al., 1991). Most other trialkylphosphines are water-insoluble and therefore odious to use. TCEP reacts with proteins and cleaves disulfide linkages leaving two free sulfhydryl groups followed by oxidation of the phosphine group. The formed bond between the phosphine and oxide is strong enough to prevent reversal reaction.

\[ R_3P + RS-SR + H_2O \rightarrow R_3P = O + 2RSH \]

Since TCEP reduces selectively disulphide bonds and no other SH-compound are added to the conjugation reaction, the conjugation can be performed without removal of excess TCEP making it attractive as a reducing agent (Hermanson 2008, Burns et al., 1991). A study of Sun et al. (2005) described how partial reduction of accessible disulphide bonds of a protein is possible. According to the study, they were able to reduce two exposed disulphide bonds of IgG1 monoclonal antibody in the hinge region by adding 2.75 fold molar excess of TCEP over the concentration of the antibody.

The picture is not available in the electronic version for reasons of copyright

Figure 4. Sulfo-SMCC conjugation. The NHS-ester of sulfo-SMCC reagent binds to primary amine group of an enzyme (or 5’ C6-NH₂ modified end of the oligonucleotide as used in this work). After purification step to remove excess sulfo-SMCC, maleimide-activated enzyme can react with sulfhydryl activated antibody and thus form a stable enzyme-antibody conjugate. (Picture: Life Technologies, 2015)
To form the proximity probe, 5’ C6-NH₂ modified end of the oligonucleotide is activated with sulfo-SMCC. Once the NHS-ester is bound with the amine group of the oligonucleotide, a purification step is performed to remove the excess sulfo-SMCC. After that, the oligonucleotides can be mixed with antibodies that have been previously treated with TCEP. At the right molar excess of TCEP ideally, only one disulfide bond at the hinge region is cleaved leaving two sulphydryl groups to react with maleimide group linked to the oligonucleotides.

Aim

The aim of the project was to develop specific assays to detect endogenous PPIs known as biomarkers of autophagy that are sensitive enough to be apply on patient samples. The assays can later be applied simultaneously to detect multiple events of autophagy on CRC tissue. This method called in situ Multiplexing can provide an important tool for scientists to deepen the understanding of different mechanisms and pathways in cells as well as the links between them.
Materials and methods

Cells

Caco-2 cells were grown in a minimum essential medium (MEM), 20 % fetal bovine serum (FBS), 0.2 mM glutamine and penicillin/streptomycin solution (PEST). For SW480 cells we used Dulbecco’s Modified Eagle Medium (DMEM), 10 % FBS, 0.2 mM glutamine and PEST. The cells were seeded on eight well slides at a seeding density of 10.10^4 cells /cm^2 for the Caco-2 cell line and 6.10^4 cells /cm^2 for the SW480 cell line. 72 hours after seeding, serum starvation and 150 mM CoCl_2 treatment was started for the next 24 h for half of the slides (hereafter referred as “treated cells”). An hour before the end of the treatment, 100 nM of bafilomycin was added to the slides with treated cells and the ones with regular growing cells (hereafter referred as “untreated cells”). The wells were washed with ice cold phosphate buffered saline (PBS) two times and then fixed with 4 % paraformaldehyde (PFA) for 15 min on ice and then for 15 min at room temperature. After fixation the wells were washed again two times with PBS. The slides were kindly prepared by my supervisor Gaëlle Cane.

Affinity probes

Our affinity reagents were the commercially available antibodies listed in the Table 1.

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<tr>
<th>Target</th>
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<th>Source</th>
<th>Product Number</th>
<th>Epitope</th>
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</thead>
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<td>LC3</td>
<td>Nanotools</td>
<td>Mouse monoclonal</td>
<td>LC3-5F10</td>
<td>N-terminus of LC3-B</td>
</tr>
<tr>
<td>p62/SQSTM1</td>
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<td>Mouse monoclonal</td>
<td>610833</td>
<td>Human p62 lck ligand aa. 257-437</td>
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<tr>
<td>p62/SQSTM1</td>
<td>Santa Cruz</td>
<td>Rabbit polyclonal</td>
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<tr>
<td>BNIP3</td>
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<td>Goat polyclonal</td>
<td>sc-1715</td>
<td>C-terminus of BNIP-3 of human origin</td>
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Validation of the assay

Validation of the probes: isRCA

The PLA probes, the antibody diluent and the blocking buffer for isRCA and isPLA assays were manufactured by Olink Bioscience. Olink is a spin-off company of Molecular Tools lab in Uppsala University where I did this degree project.
The cells were first permeabilized with 0.4 % Triton X and 0.05 % CHAPSO in PBS for 5 min, followed by 5 min washing with PBS. The cells were then blocked with the blocking buffer for 1 h at 37 °C. After blocking, primary antibodies were diluted in the antibody diluent and incubated at 4 °C overnight. The following day, the slides were washed for 1 h in tris-buffered saline (TBS), 0.05% tween-20 (TBST). Subsequently, the cells were blocked with the blocking buffer for 15 min at 37 °C. Commercial PLA primer probes (Table 2) from Olink were used as secondary antibodies. About 10 min before applying the secondary probe solution to the wells, the proximity probes were mixed with the antibody diluent according to manufacturer’s protocol. The slides were incubated with the secondary probes for 1 h at 37 °C and subsequently washed for 1 h with TBST. To hybridize the padlock to the conjugated secondary antibodies, we mixed 0.1 μM of phosphorylated padlock with the hybridization buffer A containing 0.25 mg/ml of BSA (New England BioLabs), 25 mM of NaCl and 0.10 % of Tween-20 in 1x T4 ligation buffer (Thermo Scientific). The slides were then incubated for 30 min at 37 °C followed by washing two times with TBST. To hybridize the circle forming oligonucleotides to the conjugated secondary antibodies, we mixed 0.1 μM of phosphorylated splint and 0.1 μM of phosphorylated backpiece in the hybridization buffer A and applied the solution to the wells for 30 min. The remaining steps were performed in a similar manner as described in the previous paragraph. After mounting, the slides were ready for microscopy.

Validation of the pair of probes: isPLA

The cells were first permeabilized and blocked in a similar manner than described in the previous paragraph. The primary antibodies for both target proteins were mixed in antibody diluent according to manufacturer’s protocol and incubated overnight at 4 °C. The following day, the slides were washed and blocked as previously described. The secondary probe solution was prepared by mixing both PLA primer and PLA blocked probes (Table 2) in the antibody diluent according to manufacturer’s protocol. The secondary probes were applied to the wells, incubated and washed in a similar manner as described previously. To hybridize the circle forming oligonucleotides to the conjugated secondary antibodies, we mixed 0.1 μM of phosphorylated splint and 0.1 μM of phosphorylated backpiece in the hybridization buffer A and applied the solution to the wells for 30 min. The remaining steps were performed in a similar manner as described in the previous paragraph. After mounting, the slides were ready for microscopy.

Phosphorylation of circle forming oligonucleotides

To enable the ligation of the padlock probe used in isRCA as well as the splint and the backpiece used in isPLA (Table 2), the oligonucleotides need to be phosphorylated. Phosphorylation was performed according to manufacturer’s instructions (Thermo scientific). For each phosphorylation, we mixed 10 μM of oligonucleotides, 1 mM of ATP (Thermo Scientific) and 0.25 U/μl of PNK (Thermo Scientific) in 1x PNK buffer A (Thermo Scientific) and incubated for 60 min at 37 °C. After incubation the enzyme was inactivated by incubating the solution at 75 °C for 10 min.
Table 2. Sequences of the oligonucleotides used in the experiments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3 Padlock probe</td>
<td>5’-GTTCGTTCATACAGTGAATGCAGTCCGTCTAAGAGAGTAGT</td>
</tr>
<tr>
<td></td>
<td>ACAGACGCGTCAAGAGTGTCTA 3’</td>
</tr>
<tr>
<td>S3 Splint</td>
<td>5’-GTTCGTTCATATTATTAACGCTCTTAA 3’</td>
</tr>
<tr>
<td>S3 Backpiece</td>
<td>5’-CTATTAGCGTCCAGTGAATGCGAGTCCGTCTAAGAGAGTAGT</td>
</tr>
<tr>
<td></td>
<td>ACAGACGCGTCAAGAGTGTCTA 3’</td>
</tr>
<tr>
<td>S3 Primer Probe</td>
<td>5’-AAAAAAAAAAAAATATGACAGACACTAGACACTCTTT 3’</td>
</tr>
<tr>
<td>S3 Blocked Probe</td>
<td>5’-AAAAAAAAAAAAAGACGCTAATAGTTAGAAGACGCTTUUU 3’</td>
</tr>
<tr>
<td>S3 Detection Probe</td>
<td>5’-CAGTGAATGCGAGTCCGTCT (UUUU) 3’</td>
</tr>
</tbody>
</table>

**Data analysis**

The slides were analyzed using an epifluorescence microscope (Axioplan 2, Zeizz). The microscope was equipped with AxioCam HRm (Zeizz) and a mercury lamp. For all images, filters for DAPI and Cy 3.5 were used and acquired at 40x magnification. The images were collected with AxioVision software (Release 4.8.2 SP3, Zeizz) and analyzed through the free open-source image analysis software CellProfiler (www.cellprofiler.org) (Carpenter et al., 2006).

**Probes for primary antibody-based PLA**

Both sulfo-SMCC and sulfo-SANH conjugation methods were used for primary antibody conjugation. In Table 3 are listed the conjugated antibodies together with the used conjugation method.

**Sulfo-SMCC conjugation**

The oligonucleotides were activated by mixing 2 mM sulfo-SMCC (Thermo Scientific) and 100 μM of 5’ C6-NH2 modified oligonucleotides (IDT). The mix was then incubated at room temperature for 30 min. After incubation, the excess SMCC was removed by a precipitation step. 300 mM of NaAc was mixed to the solution followed by addition of 99.6 % ice cold ethanol. The solution was incubated at -20 °C for a minimum of 30 min and afterwards centrifuges 15 min at 4 °C at maximum speed. The supernatant was discarded and 70 % ice cold ethanol was added to the tube with the pellet. The solution was centrifuges again at maximum speed for 5 min at 4 °C. The supernatant was removed and the pellet was dried. Lastly, water was added to reach the original concentration of 100 mM of oligonucleotides.

We prepared the AMICON® Ultra-0.5 filter (100K) first by adding 500 μl of 0.1 N NaOH solution to the column and then incubating the column for 5 min. Right after, the column was centrifuged 5 min at 10,000g. After that the column was washed three times by adding 500 μl of PBS followed by centrifuging 5 min at 10,000g (Eppendorf Centrifuge 5415R). 1-2 mg/ml in 50 μl of antibody solution was then added to the filter and centrifuged for 5 min at 10,000g. In case the buffer of the antibody had some unwanted substances such as glycerol, a buffer exchange step was required. The buffer exchange step was performed by adding 1:1 ratio of antibody solution and PBS to the filter followed by centrifuging for 5 min at 10,000g. Subsequently, two additional rounds of PBS were performed. Before collecting the concentrated antibody, we pipetted up and down the antibody solution in the filter in order to reach maximum yield. The collection step was performed by flipping the filter upside down and then centrifuging it for 3 min at 1,000g. The concentration was measured with Nanodrop (Nanodrop 2000, Thermo Scientific). For antibody reduction, 200 μM of
TCEP (Thermo Scientific) stock was prepared in PBS. Right after, four times molar excess of TCEP was added to the antibody solution. The mix was then incubated at 37 °C for 15 min. After incubation, the oligonucleotide-maleimide solution was mixed with the antibody solution so that the antibody-oligonucleotide ratio reached 1:6. The solution was then incubated at 37 °C for 45 min and then at 4 °C overnight.

**Sulfo-SANH conjugation**

The antibody was concentrated first following the protocol detailed before in the “Sulfo-SMCC” paragraph. Right after, sulfo-SANH powder (Solulink™) was diluted in water following the manufacturer’s protocol. The solution was then mixed with the concentrated antibody so that the molecular ratio of antibody-sulfo-SANH reached 1:25. The mixture was incubated in dark at room temperature with gentle agitation for 2.5 h. About 15 minutes before the incubation time ended, the equilibration of Zeba™ spin desalting column 7K MWCO (Thermo Scientific) was performed according to the manufacturer’s instructions. The desalting column was used to get rid of the excess sulfo-SANH as well as to change the buffer from PBS to 1x conjugation buffer containing 100 mM of NaHPO₄ and 150 mM of NaCl. The pH of the buffer was set to 6.0. Once the buffer exchange was done, the purified sulfo-SANH activated antibodies were mixed together with aldehyde-modified oligonucleotides so that the molecular ratio of antibody-oligonucleotide reached 1:3. We also added 10 mM aniline to the solution to catalyze the conjugation reaction. The solution was incubated 2.5 h at room temperature in the dark with gentle agitation. After the incubation time, buffer exchange from 1x conjugation buffer to PBS was performed using the same desalting column as described before.

**Table 3. List of conjugated antibodies, method of conjugation and oligonucleotide arms.**

<table>
<thead>
<tr>
<th>Conjugated Antibody</th>
<th>Producer</th>
<th>Conjugation</th>
<th>Arm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQSTM1</td>
<td>Santa Cruz</td>
<td>SANH</td>
<td>S3 Primer</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>Santa Cruz</td>
<td>SANH</td>
<td>S3 Blocked</td>
</tr>
<tr>
<td>LC3</td>
<td>Nanotools</td>
<td>SMCC</td>
<td>S3 Primer</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Santa Cruz</td>
<td>SMCC</td>
<td>S3 Primer</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Abcam</td>
<td>SANH</td>
<td>S3 Primer</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Abcam</td>
<td>SANH</td>
<td>S3 Blocked</td>
</tr>
</tbody>
</table>

**Purification and concentration**

The conjugates were purified using high-performance liquid chromatography (HPLC) (Äkta™ pure, GE Healthcare). We used Superdex™ size exclusion column (GE Healthcare) to separate the formed conjugates from unconjugated antibodies, oligonucleotides and remaining conjugation reagents. The flow rate of the chromatography was set to 0.5 ml/min and direction of the flow was set to up flow. For monitoring the run we used UNICORN software (GE Healthcare).

After the purification, the fractions of each peak were collected and concentrated with AMICON® Ultra-0.5 filter (100K) following the same protocol as described before in the “Sulfo-SMCC conjugation” paragraph. Each purified and concentrated fractions of primer arm-conjugated antibody were tested first using isRCA and later together with each fractions of blocked arm-conjugated antibody using isPLA.
Results

Validation of the probes

We decided to focus on the interactions between LC3 and SQSTM1 as well as the interaction between BNIP3 and Bcl-2. To find the most suitable antibodies for our experiment, we investigated the possible antibodies and ended up choosing the group of antibodies that is listed in Table 1. There were several factors that influence our decision. Previous experiments have shown that some companies offer more desirable antibodies for conjugation. Some antibodies are easier to conjugate, do not contain undesirable reagents in the buffer that could interfere with the conjugation reagents or bind more specifically. What is important in isPLA using secondary probes is that the two antibodies used in the assay are from different species so each probe binds only one primary antibody. Though, once the antibodies are conjugated, it does not matter whether they are raised in the same specie since secondary antibodies are not used in the assay. Moreover, the combination of antibody pair has to be taken into consideration as they should not compete and each epitope has to be accessible while the proteins are fixed in the complex. Another important factor when choosing the antibodies is that they are tested for appropriate applications. Preferably the antibodies have been tested using immunohistochemical assays on frozen or paraffin embedded tissues or alternatively by immunofluorescence. Also supporting publications of the experiments or earlier reviews of the product are taken into consideration. Good investigation before purchase is important in order to save time and money.

Based on the manufacturer’s recommendations, we set up a range of dilutions to test the sensitivity of the antibodies as well as the specificity. The optimal dilution was first set for isRCA using slides with untreated colorectal cancer cells (Table 4).

Table 4. List of the antibodies and their optimized dilutions for isRCA.

<table>
<thead>
<tr>
<th>Target</th>
<th>Producer</th>
<th>Host</th>
<th>Optimized dilution (isRCA)</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC3</td>
<td>Nanotools (N)</td>
<td>Mouse</td>
<td>1/400</td>
<td>Good</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>BD Bioscience (BD)</td>
<td>Mouse</td>
<td>1/1000</td>
<td>Good</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>Santa Cruz (SC)</td>
<td>Rabbit</td>
<td>1/2000</td>
<td>Bad</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Santa Cruz (SC)</td>
<td>Goat</td>
<td>1/200</td>
<td>Bad</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Pierce (P)</td>
<td>Rabbit</td>
<td>1/500</td>
<td>Good</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>BD Bioscience (BD)</td>
<td>Mouse</td>
<td>-</td>
<td>Bad</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Abcam (A)</td>
<td>Mouse</td>
<td>1/500</td>
<td>Good</td>
</tr>
</tbody>
</table>

The optimal primary antibody concentration in the isRCA assays was around 0.2-2 μg/ml. The amounts were in a reasonable range since the required concentration can rises up to 10 μg/ml. After isRCA, the combination of antibody pairs targeting the interactions Bcl-2-BNIP3 and LC3-SQSTM1 were tested and optimized by applying isPLA using both untreated and treated cells (Table 5). This model served as a biological control for the specificity of the assays. A variation of signal between treated and untreated cells following what is expected according to the literature would support that the assays detect the targeted interactions. An experimental control where the primary antibodies were omitted was performed in parallel of every isRCA experiment.
As a starting dilution for the antibody with the weakest efficiency within a pair according to the isRCA experiments, we used a dilution that was twice of the optimized one. For the binding partner, we tested a few different dilutions. This procedure was done to obtain the highest possible amount of binding events and thus detect as many interactions as possible. The experimental control was performed as described previously. We also wanted to investigate the specificity of the binding; the subcellular localization was quite encouraging since the signal is mostly located in the lysosome-rich perinuclear area that surrounds the nucleus as expected according to the literature (Figure 5).

Table 5. List of antibody pairs optimized by isPLA.

<table>
<thead>
<tr>
<th>Primary Antibody 1</th>
<th>Dilution</th>
<th>Secondary Antibody 1</th>
<th>Primary Antibody 2</th>
<th>Dilution</th>
<th>Secondary Antibody 2</th>
<th>PPI Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2 (A)</td>
<td>1/100</td>
<td>Mouse</td>
<td>BNIP3 (P)</td>
<td>1/50</td>
<td>Rabbit</td>
<td>BB1</td>
</tr>
<tr>
<td>Bcl-2 (A)</td>
<td>1/100</td>
<td>Mouse</td>
<td>BNIP3 (P)</td>
<td>1/100</td>
<td>Rabbit</td>
<td>BB2</td>
</tr>
<tr>
<td>Bcl-2 (A)</td>
<td>1/100</td>
<td>Mouse</td>
<td>BNIP3 (SC)</td>
<td>1/50</td>
<td>Goat</td>
<td>BB3</td>
</tr>
<tr>
<td>LC3 (N)</td>
<td>1/100</td>
<td>Mouse</td>
<td>SQSTM1 (SC)</td>
<td>1/500</td>
<td>Rabbit</td>
<td>LS1</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>Mouse</td>
<td>-</td>
<td>-</td>
<td>Rabbit</td>
<td>NCR</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>Mouse</td>
<td>-</td>
<td>-</td>
<td>Goat</td>
<td>NCG</td>
</tr>
</tbody>
</table>
(The figure continues on the next page)
We were also interested whether the amount of the signal varied between untreated and treated cells. In treated cells, the autophagy machinery should be induced by the depletion of nutrients and hypoxia whereas in untreated cells autophagy should only work as a basal level removing the damaged proteins and organs. The taken pictures of the PPIs were analyzed with CellProfiler and the average amounts of blobs per cell were compared between treated and untreated cells and normalized to the untreated condition (Figure 6). As the signal is notably higher in treated cells compared to untreated cells for both interactions and with every assays tested, it is likely that the PPIs were detected specifically and thus, the assays have been validated at this stage for further development.

Figure 5. IsPLA detection of PPIs between Bcl-2 and BNIP3 as well as between LC3 and SQSTM1 using treated and untreated Caco-2 cells. The codes used in the pictures indicate a certain PPI and specific antibody dilutions. BB1 and BB2 stands for Bcl-2 (A)-BNIP3 (P) interaction, BB3 stands for Bcl-2 (A)-BNIP3 (SC) interaction and LS1 stands for interaction between LC3 (N) and SQSTM1 (SC). NCR and NCG are negative controls. More detailed information including the dilutions and codes can be found in Table 5. The white arrows in the pictures indicate the location of the blobs in the lysosome rich-perinuclear area. The contrast of the pictures has been consistently modified to make the blobs more visible for this manuscript.
Once we had screened all the antibodies and found the most suitable ones to use as an assay, they were conjugated using either sulfo-SMCC or sulfo-SANH conjugation. Both, priming and blocked probes were generated for each antibody as a variation of efficiency has been previously noticed once the probes were applied in PLA assays. The antibody directed against LC3 and BNIP3 were conjugated using the sulfo-SMCC method that targets the sulfhydryl groups of antibodies, and more specifically the exposed one in the hinge region, and thereby, does not affect the specificity and affinity of the variable domain. Thus this method is preferred but still in development. For this reason, the antibody directed against SQSTM1 and Bcl-2 have been conjugated with the commonly used sulfo-SANH method known to potentially deteriorate the variable domain of the antibody affecting its efficiency and specificity. After the conjugation was performed, the samples were purified using HPLC that fractionates the different components according to their size. Depending on which conjugation method was used we got different chromatograms following a same principle. The biggest molecules - here the conjugated antibodies - are eluted first, following by the unconjugating ones if any and finally, the low molecular weight unconjugated oligonucleotides. Sulfo-SMCC conjugates gave a graph that had several peaks (Figure 7) suggesting different level of conjugation whereas the purification of the antibody directed against Bcl-2 by sulfo-SANH gave a graph that had only two peaks (Figure 8). Even though it is well-known that proteins absorb UV light near spectrum of 280 nm whereas oligonucleotides have absorbance around spectrum of 260 nm, it is difficult to predict the composition of each fraction as the linker resulting from the chemistry of conjugation affects the overall absorbance of the antibody and modified
oligonucleotides (Noble, 2014, Bonilla et al., 2011). By running each form of the antibody and oligonucleotides independently, we could predict that the highest peak eluted after about 17 ml is the free oligonucleotides after chemical modification. The antibodies were eluted after about 13 ml suggesting that the very first fractions eluted from 8 ml to about 10 ml contains the conjugated antibodies (Figure 7).

Figure 7. Chromatograph of sulfo-SMCC conjugated BNIP3 (SC) antibody. The figure demonstrates a trend of chromatograph obtained when running antibodies conjugated by sulfo-SMCC.
We collected and combined the fractions of each peak and concentrated them (Table 6). After concentration step the concentration of peak solution was tested using Nanodrop spectrometer. The concentrated fractions that seem to contain conjugates were stored and tested to assure that the conjugate functions also in situ.

Table 6. List of conjugated antibodies and their respective starting volumes, initial and final concentrations after HPLC purification.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Arm</th>
<th>$V_{ab}$ after concentrating (μl)</th>
<th>$C_{ab}$ after concentrating (mg/ml)</th>
<th>Peak Fractions</th>
<th>$C_{ab}$ after HPLC (mg/ml)</th>
<th>IsRCA works</th>
<th>IsPLA works</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC3 (N)</td>
<td>Primer</td>
<td>31</td>
<td>0.9</td>
<td>F1</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F2</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F3</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F4</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SQSTM1 (SC)</td>
<td>Primer</td>
<td>90</td>
<td>2.5</td>
<td>F1</td>
<td>1.15</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F2</td>
<td>0.90</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>F3</td>
<td>0.70</td>
<td>-</td>
<td>No</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F4</td>
<td>0.85</td>
<td>-</td>
<td>Yes</td>
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<tr>
<td>SQSTM1 (SC)</td>
<td>Blocked</td>
<td>110</td>
<td>2.0</td>
<td>F1</td>
<td>0.05</td>
<td>Yes</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F2</td>
<td>0.50</td>
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<td></td>
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<td></td>
<td>F3</td>
<td>0.22</td>
<td>No</td>
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<td></td>
<td>F4</td>
<td>0.00</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>BNIP3 (SC)</td>
<td>Primer</td>
<td>93</td>
<td>1.7</td>
<td>F1</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
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<td></td>
<td></td>
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<td>F2</td>
<td>0.06</td>
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<td>-</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>F3</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bcl-2 (A)</td>
<td>Primer</td>
<td>40</td>
<td>1.0</td>
<td>F1</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>F2</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bcl-2 (A)</td>
<td>Blocked</td>
<td>40</td>
<td>1.0</td>
<td>F1</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F2</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 8 The chromatograph of sulfo-SANH modified Bcl-2 (A) antibody. The figure demonstrates the general trend of chromatograph obtained when running sulfo-SANH modified antibodies.
After HPLC purification and subsequent concentration we had a sufficient yield of primer and blocked probes anti-SQSTM1 and primer probe anti-BNIP3. We tested all fractions separately. The primer probes conjugated with the priming arm were tested first using *is*RCA and later together with the blocked probes by *is*PLA. The fraction 1 and 2 of the primer probe anti-SQSTM1 generated a reasonable signal by *is*RCA. We tested both fractions of the blocked probe anti-SQSTM1 together with each fraction of the primer probe using *is*PLA. However, we could only detect the signal when using the fraction 2 of the blocked probe suggesting that this fraction contains efficient conjugated antibodies (Figure 9). The signal strength of each condition was quantified with CellProfiler. The average amount of blobs per cell generated by the combination of the fraction 1 of the anti-SQSTM1 primer probe and the fraction 2 of the blocked probe was 7.8 whereas with the fraction 2 of the anti-SQSTM1 primer probe in combination with the fraction 2 of the blocked probe, the amount was 1.9 blobs per cell (Negative control 0.2 blobs/cell).

All four fractions of primer probe anti-BNIP3 were tested using *is*RCA. A reasonable signal was only detected in the first fraction (F1) where as in other fractions (F2, F3, F4) the signal was either a very weak or there were no signal at all (data not shown).
Discussion

CRC is one of the most diagnosed and lethal cancers in the world. It is extensively studied over the years but still more knowledge is needed to understand how this cancer develops and acts in order to provide improved treatments. In the recent years, autophagy mechanism has been strongly linked to development and progression of cancer. Autophagy is an important regulator of cellular homeostasis. It degrades damage proteins and organelles and helps the cell to survive under metabolic stress. In cancer cells, the metabolic stress in increased significantly and thus autophagy is induced to provide enough nutrient and energy for the cancer cells. Autophagy seems to be a fundamental process for cancer cell. Most currently used methods to study autophagy in cell lines or tissues are either unreliable or not sensitive enough and most of them are not applicable to patient samples. Therefore, improved methods are required since they might provide new insight to develop more effective treatments against CRC. One possible method is to use isPLA. IsPLA enables specific detection of PPIs since it uses dual recognition of the target molecule. The method also provides a sensitive detection of the PPIs since several fluorophore labeled oligonucleotides can bind the RCP and thus form a distinct spot on top of the target molecule.

In this study we developed assays to detect PPIs events occurring during autophagy. We decided to focus on two of them: an interaction between LC3 and SQSTM1 located on the phagophore membrane and an interaction between BNIP3 and Bcl-2 that occurs during hypoxia-induced autophagy. These interactions were chosen based on earlier studies related to autophagy and CRC. The antibodies we chose were first tested by isRCA. We wanted to see that the antibodies bind efficiently and specifically and thus the blobs are found in the cytoplasm. With some antibodies unspecific binding was an issue. For example both Santa Cruz antibodies gave a strong signal but some of the blobs were located in areas that binding should not occur such as in between the cells. With the Bcl-2 antibody from BD Bioscience we could not differentiate between different dilutions and therefore decided not to use it in later experiments. All other tested antibodies seem to bind specifically and gave reasonable signal strength.

Both in isRCA and isPLA experiments, we aim to reach a maximal detection of PPIs events and the amount of unspecific binding was minimal. In isPLA experiments the signal seemed specific and the blobs were mainly found in lysosome-rich perinuclear area that surrounds the nucleus (Lai et al., 2014). To improve the cell models we could have omitted the bafilomycin treatment and try to detect the endogenous level of PPIs without accumulation of the autophagic vesicles. In that way we could have been closer to the level of autophagy in patient samples.

Both PPIs optimized in isPLA gave promising results. The detection of the interaction between Bcl-2 (A) and BNIP3 using the BNIP3 antibody from Pierce seem to be slightly stronger than the detection of the interaction between Bcl-2 (A) and BNIP3 performed with the Santa Cruz antibody thus being the primary choice for conjugation. The signal in both interactions was detected in the perinuclear area of the cells and the used concentrations were still in a range where they could be increased for later studies in tissue sections. However, with the antibody anti-BNIP3 from Pierce, the later optimization of the assay for tissue has more flexibility since even with 1/100 dilution we received good amount of blobs. Also the detection of the interaction between LC3 (N) and SQSTM1 using the antibody from Santa Cruz seems to be specific and had a reasonable signal density. We could not test the other SQSTM1 antibody from BD Bioscience using isPLA since like the antibody against LC3 it was also raised in mouse. According to recent publications it seems to
work well in immunohistochemistry therefore we decided to conjugate it without previous isPLA optimization.

When comparing the amount of blobs between the treated and untreated cells we could clearly observe a trend indicating that treated cells, expressing higher levels of autophagy, had distinctly more signal detected compared to untreated cells. When comparing the two interactions with the anti-Bcl-2 and anti-BNIP3 antibodies we could see that the greater difference in average amount of signal per cell was found with the antibody anti-BNIP3 from Pierce. This antibody might have a higher affinity to its target and therefore we can obtain more significant difference in the signal amount. With the LC3-SQSTM1 interaction the difference between treated and untreated cells was smaller but still the difference was clearly visible. The antibody anti-SQSTM1 from Santa Cruz had more unspecific binding compared to the one from BD Bioscience and therefore we could expect that a greater difference would have been obtained if we could have used the antibody anti-SQSTM1 from BD Bioscience. The results from the isPLA experiment indicate that both developed assays to detect these PPIs are suitable for further investigation.

As a primary conjugation method we wanted to use sulfo-SMCC mainly because it links the oligonucleotides to the sulfide group of the hinge region in the constant domain. Therefore the oligonucleotides cannot affect the antigen-binding site and thus the antigen recognition. With the sulfo-SANH conjugation it is possible that the oligonucleotide binds to NH$_2$ groups of the variable region and affects the binding affinity and specificity. However, we did not receive the expected arms on time and therefore Bcl-2 and SQSTM (SC) antibodies were conjugated with sulfo-SANH method. Both conjugation methods seem to work in the condition tested. The chromatograph of the sulfo-SMCC contained several peaks most likely because there were different amount of oligonucleotides bound to one antibody or TCEP had cleaved several disulfide bonds dividing the antibody into smaller fractions. Though, a SDS-PAGE analysis showed that no cleavage occurred for these conjugates (data not shown). The chromatograph of sulfo-SANH only had two peaks but this was most likely because all the oligonucleotides were bound to the antibodies.

We had problems to concentrate some conjugates after HPLC purification. The antibodies that had high concentration and volume after the first concentration gave a reasonable yield after HPLC purification and subsequent concentration whereas the antibodies with low starting concentration and volume barely had any antibodies left. For example if we compare the concentration and volume of the antibody anti-LC3 after first concentration step to the concentration and volume of the antibody anti-BNIP3 (SC) we can see that both the concentration and the volume is about half what the antibody anti-BNIP3 had. We aim to have at least 50 μ of 2 mg/ml antibody solution at the beginning. With LC3 and Bcl-2 we could not reach these values. It is possible that the low starting concentration makes it more difficult to reach a good yield.

We detected a reasonable signal by isPLA with the primer probe anti-SQSTM1 and the fraction 2 of the blocked probe. According to CellProfiler analysis the signal generated by the combination of the fraction 1 of the primer probe and the fraction 2 of the blocked probe was clearly stronger than the one obtained with the pairing of the fraction 2 primer probe and the fraction 2 blocked probe. Interestingly, the most efficient primer probe was the fraction 1 which did not give any signal with the blocked probe. The weaker fraction from the primer probe, the fraction 2, generated however some signal. The results are slightly controversial. Possibly the amount of oligonucleotides bound to the antibodies as well as the binding location may affect its properties.
As a conclusion, we were able to initiate the development of assays to detect biomarkers of autophagy and generate direct probes for most of them. Both conjugation methods, sulfo-SMCC and sulfo-SANH seem to work equally in the condition tested. Further controls should be done before giving any conclusion on the specificity of the probes. One of the most reliable controls is to test these probes on a cell line not expressing the target of interest endogenously or by knock out with siRNA for example. In the future the conjugation and validation of the probes will continue in a similar manner in more challenging conditions in order to test the sensitivity and specificity of detection. To do so, the bafilomycin treatment will be omitted in order to detect the endogenous level of autophagy without inhibition of the degradation process induced by this chemical compound. Then, the serum starvation and CoCl$_2$ treatment will be reduced to challenge the limit of detection of the autophagic-dependent PPIs. Once the testing probes will be completed on cell lines, the assays will be applied to colorectal cancer tissue and be optimized for it. If all goes well, these antibodies can be used to set up a multiplexed version of $i$SPLA to study several PPIs simultaneously.

The aim of the $i$SPLA multiplexing is to enable study of several pathways and mechanism in a complex organ tissue in parallel to understand the link between them and to investigate the interplay between distinct cell types. In 2013 Ke et al. presented a method were $i$n $s$itu sequencing was used for parallel target analysis of short RNA fragments in preserved tissue and cells. They developed two target approaches. For the first one they sequenced a stretch of four different nucleotides straight in an RNA transcript by gap-targeted sequencing (gap targeted approach) while in the other one the four-nucleotide stretch was placed in the padlock probe (barcode targeted approach). In both methods messenger RNA (mRNA) was first copied to complementary DNA (cDNA) and subsequently degraded followed by hybridization of padlock probe to the remaining cDNA strand. In the case of gap-targeted sequencing, the padlock hybridizes on either side of the target bases in cDNA, which is followed by gap filling by DNA polymerization and DNA ligation to enable formation of a DNA circle. For barcode-targeted sequencing, both ends of padlock bind to adjacent hybridization regions as described before, followed by a ligation step to form a circular DNA. The formed DNA circles are amplified by RCA. After RCA, anchor primers are hybridized next to target sequences on the RCP. The four interrogation probes consisting of one fixed base position (A, C, G or T) and eight random positions (N) are then added. Each library of these 9-mers is labelled with a specific fluorescent dye (Cy5, FITC, Texas red and Cy3) based on which fixed base they are carrying. The best matching interrogating probes will hybridize to the target sequence, followed by a ligation step. Each of the RCP products is seen in a certain color depending on the match base. Once being imaged, the interrogation probes are cleaved before addition of the next interrogation probe targeting the second base. The steps (ligation, imaging and cleaving) are repeated until all four target bases are read. By these four bases it is possible in theory to read up to $4^4 = 256$ different sequence barcodes (Ke et al. 2013).

The current design of $i$n $s$itu Multiplexing uses primary antibodies in which a specific oligonucleotide sequence is conjugated. The binding of probe to its target will be detected using a similar method that the barcode targeted approach. For each PPI, there will be a backpiece containing a unique four bases target sequence. The RCP attached to the protein complex will give a certain signal in each round of detection as described earlier. After all detection rounds the PPIs can be located in the tissue based on the detected four signals on one spot. To study the autophagy mechanism CRC we desire to detect several events in the mechanism at the same time. The method can even be used to understand the relationship between several different pathways and mechanism. This multiplexed version of $i$SPLA could potentially be applied in clinic for diagnostic and prognostic.
References


Glick D, Barth S, Macleod KF. 2010. Autophagy: cellular and molecular mechanisms. Journal of Pathology 221: 3-12


Wu AM, Kenanova VE, Olafsen T. 2010. Human Protein Scaffold with Controlled Serum Pharmacokinetics. Publication number: WO 2010118169 A2

