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Evaluation and development of reagents and improved protocol for flow cytometry readout using *in situ* PLA

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Abstract

The diagnosis of cancer today is obsolete, depending upon pattern recognition and non-quantifiable data. The time consuming diagnosis is often performed on biopsies, fixed using non standardised procedures, and leaves room for dubious results. The diagnosis is also invasive, exposing patients to risk of infections and discomfort due to the need of tissue samples. The knowledge about changes in protein expression levels related to cancer can instead be utilized to generate a new diagnostic tool. By adapting the *in situ* proximity ligation assay (*in situ* PLA) to cells in solution, it is possible to detect proteins, or protein interactions, within cells without the need for tissue samples. Since the method is both highly sensitive and specific, it delivers reliable results. In this report, the *in situ* PLA method for cells in solution is combined with flow cytometry readout. Hence, a new and less invasive diagnostic tool for cancer, delivering highly accurate high throughput single cell analysis, may be on the rise.

Keywords

In situ PLA, flow cytometry, diagnostic method, cancer,

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Populärvetenskaplig sammanfattning

Korrekt sammansättning och uppbyggnad av proteiner är avgörande för möjligheten till liv. Konstruktionen av kroppsdelar, kommunikation mellan och i celler samt enzymatiska reaktioner, alla funktioner drivs av proteiner. Betydelsen av ett felfritt bildande av dessa molekyler är stor, och avgör proteiners funktion i en organism. Fel uppbyggnad eller sammansättning resulterar ofta i allvarliga sjukdomar. Cancer, en sjukdom som drabbar många, är en följd av funktionsförändring hos vissa proteiner, vilket påverkar mängden av andra proteiner. Genom att tidigt kunna identifiera vilka proteiner som finns i över- eller underskott kan vi också tidigt starta behandling mot sjukdomen, och på så vis också öka chansen för överlevnad.

In situ proximity ligation assay är en metod vilken kan identifiera proteiner och synliggöra dem. Genom att markera varje protein av intresse med en lysande signal, kan man enkelt beräkna den exakta mängden av specifika, detekterade, proteiner i varje cell.

Det här projektet går ut på att möjliggöra utförandet av *in situ* PLA på celler i lösning, och att därefter avläsa mängden signaler i varje cell med hjälp av flödescytometri. Detta för att utveckla en metod som snabb och exakt kan ställa en cancerdiagnos.

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1 List of abbreviations and terminology

Affinity	Strength with which a molecule interacts with a specific target. High affinity means strong interaction
Antigen	Target molecule for antibodies
Far Red	Fluorophore which is being excited at 594 nm and emits light at 624 nm
Fluorophore	Molecule that is excited by light at a specific wavelength, and through relaxation emits light at a longer wavelength (Rayleigh scattering)
FNAB	Fine needle aspiration biopsy
Histopathology	The search for disease through studies of tissue
IF	Immunofluorescence
IHC	Immunohistochemistry
ImRCA	Immuno rolling circle amplification
In situ	Inside cells
Orange	A fluorophore which is being excited at 554 nm and emits light at 579 nm
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PLA	Proximity ligation assay
Primary antibody	Antibody targeting molecule of interest
RCA	Rolling circle amplification
Secondary antibody	Antibody targeting primary antibody bound to target of interest
U937	Cell line consisting of human histiocytic lymphoma cells

2 Introduction

The diagnosis of cancer is today a time consuming event that mainly is based on the structural recognition of carcinogenic cells, as well as investigations of expressed amount of certain proteins. Experienced pathologists are needed to interpret the sometimes ambiguous results, and no standard for sample treatment has yet been established. Hence, the method for diagnosis of cancer leaves room for optimizations.

This project has focused on the development of an alternative way of diagnosing cancer. By improving the analysis of protein detection, the new diagnosing method can deliver more quantitative data under a much shorter period of time.

By defining what cancer is, and how the diagnosis of the disease is performed today, this introduction aims to clarify the possibilities, as well as necessities, for improvements. Also, the introduction describes the techniques involved in the new workflow for diagnosis of cancer.

2.1 The cause, and diagnosis, of cancer

Every living cell contains the recipe of life, also called the genetic material. Like a cookbook the genetic code is a composition of different smaller recipes describing specific designs of components that collaborate with each other. It is collaboration that makes life possible.

2.1.1 Protein function is of importance

A large part of the coding genetic material is translated into proteins, macromolecules with a wide variety of functions. Some proteins are involved in the construction of an organism. Other proteins take part in the communication network exploited by cells, in the immune response and function as catalysts driving important reactions. Since proteins of different kinds are involved in almost every event taking place in living organisms, their collaboration, as well as their construction, is of vital importance. The large variation, as well as the amount of molecules, enables a complex life. But the complexity, however, also expands the possibilities for fatal deviations.

Proteins consist of specific combinations of the commonly existing 20 amino acids (1). The amino acid arrangement contributes to the specific folding of every protein, and gives them the specific characteristics that define that exact protein class (2). Proteins, as a group, can be seen as the most versatile molecules in a cell, and the biochemical functions, possessed by these adaptable macromolecules, are the basis of the cellular functions performed by the proteins.

The interactome, all molecular interactions within a cell, in humans is estimated to include around 650 000 protein interactions (3), a stunning number that helps one grasp the extent, and also the importance, of protein collaboration. With a large number of interactions taking place, possibilities for inaccurate communications between proteins expand. An incorrect folding of a protein can be caused by faulty posttranslational modifications or mutations, and may contribute to a biochemical function that deviates from the protein's normal function (4). A change in the biochemical function of the protein may also alter the cellular function, something that may affect the cells behaviour. Events taking place due to changes of protein function is up or down regulation of protein expression, and a well-known disease that is caused by an altered protein expression is cancer.

2.1.2 Cancer, and how we are diagnosing it

Cancer occurs when a previously healthy cell is losing control over its own cell division (5). Usually this division is a strictly controlled process organized by a number of proteins but when mutations occur in the specific genes coding for the proteins, changes in the expression of the molecules are prone to follow. Alterations in protein levels may then result in the inability to regulate the cell division (5). Usually mutations urges the cell to perform programmed cell death, also called apoptosis, but some alterations in the genetic material may do just the opposite. Instead of programmed cell death, the cell goes into an immortal state. Those alterations, causing immortality and / or uncontrolled cell division, are considered cancerous mutations (6). This disease affects a large part of the humanity. During the year of 2007, 50 100 people were diagnosed with cancer in Sweden alone (7).

Much research has been done, and much is still being explored, concerning cancer. Today we are learning more and more about what types of mutations, and thereby what types of protein alterations, that give rise to specific types of cancer. The growing knowledge is creating new abilities for us to diagnose, and treat, a wide variety of cancer diseases. However, despite the growing knowledge and understanding of the disease, we are using diagnostic methods that are obsolete.

In order to diagnose a patient with cancer today, invasive diagnostic methods are being used. When a doctor suspects that his or her patient is suffering from cancer, a biopsy may have to be performed. The sample, a bit of tissue suspected to contain carcinogenic cells, is being fixated in order to preserve the cells. After fixation, a pathologist screens the tissue for cancerous cells, a procedure called histopathology (8). When looking for carcinogenic cells, the pathologist is looking at the morphology of the cells in the fixed tissue. In order to easier visualize individual cells in the sample, the tissue is stained using different standard techniques. The nucleus may be visualized using dyes that stain the chromatin, and the cytoplasm is then stained with a contrasting dye (9). If wanted, specific targets such as particular proteins may also be visualized. For this purpose staining methods that exploits antibodies, targeting wanted molecules, are being used.

Today biopsies are fixated using formalin, and are thereafter embedded in paraffin. This method, resulting in formalin-fixed paraffin-embedded (FFPE) tissue, has been used for over 100 years (10). However, no uniform standard of how to perform the fixation exists, and different laboratories may execute the method differently. Even though the fixation may have been performed in an incorrect way, or the tissue is poorly fixated, pathologists are allowed to perform cancer diagnoses (10). This permits for deviation in results without considering variation in sample preparation.

The deviating results do contribute to the non-quantifiable outcomes resulting from histopathology. This means that pathologists today must base their diagnosis on pattern recognition and fully trust upon their experience since no quantitative results are available and used methods are hardly reproducible (10). In addition to pattern recognition, pathologists today also use immunological assays to visualize the amount of specific carcinogenic markers, such as Human Epidermal growth factor Receptor 2 (HER2), as well (10). This choice of protein detection method does not provide quantitative data.

With regards to the above arguments it can be concluded that the usage of the, somewhat obsolete, FFPE tissue gives results that are not totally trustworthy. Due to non-standardised procedures generating FFPE tissues in various conditions, resulting diagnosis may be questioned. No comprehensive quality control, nor quality assurance, are done on the resulting tissues, and we do not know for sure that the method for tissue preservation does not impinge on any proteins (11). Despite this, immunological assays are being used for diagnostic purposes on FFPE tissues.

Hence, we are able to diagnose cancer today. The workflow that is being used gives us the possibility to distinguish carcinogenic cells from normal cells, but it is not without issues. The necessity to fixate cells before investigating the morphology may affect the results. Also, the methods used for detection of specific molecules do have some limitations and drawbacks.

2.2 Protein detection methods, and their results

Depending on choice of protein detection method used in the diagnosis of cancer, the results may deliver different types of information. When choosing protein detection method for the purpose of diagnosing cancer, drawbacks of the methods must be taken into consideration. Today, used methods deliver non quantifiable data, complicating the manually performed analysis.

2.2.1 Immunohistochemistry and immunofluorescence

Immunohistochemistry (IHC) and Immunofluorescence (IF) are two commonly used dying techniques, visualising specific proteins in cells, when diagnosing cancer (12). Both methods use antibodies to direct reagents to specific targets. By utilizing the antibodies natural affinity towards specific molecules, the methods can visualize molecules of interest in a precise manner.

IHC is a method that can exploit enzymatic reactions in order to visualize specific antigens inside cells (in situ). An indirect IHC utilizes two antibodies. Here a primary antibody targeting a protein, antigen, of interest, is used. Throughout the antibody´s natural affinity towards a specific epitope, a location on the protein of interest, the antibody may bind to the antigen. Using a secondary antibody that is targeting the Fc region, a non-variable region, on the primary antibody and also has an enzyme conjugated to it, a visualization of the protein target is possible. A specific substance may now be added. When this specific substance comes in contact with the enzyme, conjugated to the secondary antibody, the enzyme recognizes the substance and converts it to a specific product. This product can now be detected as a colour using a bright-field microscope (13). The full sequence of events can be seen in figure 1.

Figure 1. *A schematic description of indirect IHC. A primary antibody targets the protein of interest. To the primary antibody a secondary, with an enzyme conjugated to it, antibody is bound. The enzyme converts substrates to coloured products. Picture used with full permission from Olink Bioscience.*

By exploiting the colouring of the products, IHC can visualize wanted proteins. Since pathologists are using the method to investigate how much of specific proteins that subsists in cells, rather than visualizing whether the protein of interest is present or not, the analysis of the results becomes non-precise (11). This since the amount of protein directly translates into the amount of colouring of the sample, something that is analysed by eye. Since it is impossible to detect one or a few visualised proteins, due to the minor colouring, it is not possible to state how manny proteins that has been visualised. No standard reference material is available today and thereby there is no universal material that is indicating colouring of healthy cells or tissues and can be used to compare investigated sample with (11). Therefore a minor increase or decrease of protein may go undetected. Furthermore, due to this the reproducibility is poor.

Other drawbacks using the IHC method is potential risk for cross reactivity of the antibodies (14), meaning that the antibodies used have affinity towards more proteins than just the target of interest. This potential for cross reactivity generates the possibility for false positives, meaning that a stronger colouring of the sample may occur in contrast to the amount of protein that is present. Beside this shortcoming, it is also hard to detect low abundant antigen using this method (14). Small amounts of proteins only gives rise to a minor colouring that is hard to distinguish. Hence, small amount of the target protein may be perceived as a negative result.

There are also other immunological assays that are used to visualize interesting proteins in situ when diagnosing cancer. Instead of using colouring detected in a bright-field microscope, fluorescence is utilized. IF is a method that employs fluorescently labelled antibodies to detect, and visualize, specific antigens. Indirect IF uses a primary antibody that has a high affinity towards an epitope positioned on the protein of interest. To the primary antibody a secondary antibody is directed. This secondary antibody has a fluorophore conjugated to it, allowing for detection and visualization using a fluorescence microscope, i.e. excitation source, emission filter and a detector (15). A schematic drawing of the method can be seen in figure 2.

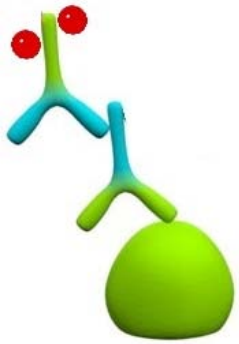


Figure 2. *A schematic description of indirect IF. A primary antibody targets protein of interest. A secondary antibody, with fluorophores conjugated to it, bound to primary antibody. Picture used with full permission from Olink Bioscience*

Fluorescence is a process where molecules are excited by light of a certain wavelength and then emits light in another wavelength. This is a naturally occurring process. This means that when exciting fluorophores conjugated to antigen bound antibodies, you may also risk exciting other molecules within the cell. This event can make it more difficult to detect the target protein, and also makes it more difficult to detect lower concentrations of protein (15). As for IHC, the specificity of this method is also dependent on the primary antibody´s affinity towards the target protein. Also here the risk for cross reactivity exists (15), which enables for false positives. Apart from this, resulting signals from IF are prone to be photobleached (15), meaning that the fluorophore is being destroyed when exposed to light throughout a longer period of time.

Hence the diagnostic methods used do not meet the standards of today. Performing IF and/or IHC on FFPE tissues do give experienced pathologists a hint of whether tissues might contain carcinogenic cells or not. But in order to deliver quantifiable results, new diagnostic methods need to be developed, and problems concerning FFPE tissues have to be overcome. The human factor, specifically the diagnosis which partly is done by the pathologist's perception of more or less colouring/fluorescence, should preferably be reduced. A new, more specific and reproducible method would help improving the diagnosis of cancer.

2.3 An alternative method for diagnosing cancer

In order to improve the diagnosis of cancer, one would wish to increase the sensitivity as well as the selectivity of the used protein detection method(s). In contrast to IHC and IF, in situ Proximity ligation assay (in situ PLA) is able to deliver more specific and quantitative results since many of the drawbacks of the standard methods have been compensated for (16). The method is currently performed on cells fixated onto a glass slide, or on FFPE tissues. However, by modifying the current protocol so that in situ PLA can be performed on fixed cells free in solution, an analysis using flow cytometry is possible. This combination of method would enable a fast and accurate diagnostic method, giving quantifiable results.

2.3.1 In situ PLA

In situ PLA is a fairly new method that is used to, for instance, detect proteins or protein interactions (17). Just as IHC and IF, *in situ* PLA uses antibodies to detect the molecule of interest. However, the

risk for cross reactivity is greatly reduced by the requirement of antigen bound antibodies in close proximity (18). Instead of utilizing one epitope on the molecule of interest *in situ* PLA uses two epitopes in close proximity to each other. To the chosen sites, two different antibodies, bind in. To the bound primary antibodies two different proximity probes are directed (18). A proximity probe consists of an antibody with a conjugated oligonucleotide to it. The secondary antibodies, constituting a part of the proximity probes, are targeting one primary antibody each (18). When all of those bindings have occurred in the designed way, the oligonucleotides conjugated to the secondary antibodies will end up in close vicinity to each other. When the proximity probes have bound to their target, new single stranded (ss) oligonucleotides are added. Parts of the ss oligonucleotides may, due to the closeness of the proximity probes, hybridize to the ss oligonucleotides conjugated to the probes (18). Using T4DNA ligase, the hybridized ss oligonucleotides are ligated, and they are now forming a ss DNA circle that is partially hybridized to the proximity probes (18). Phi 29 DNA polymerase, a polymerase with a strong strand displacement capacity, will now be able to amplify the circle in an event called rolling circle amplification (RCA). Due to the strong strand displacement capacity of phi 29, the polymerase will be able to amplify the circle in a large amount of copies (18). The product, repeated copies of the ss oligonucleotide circle, is called the RCA product. Since the RCA product has a known, repeated, sequence it is possible to direct one type of shorter ss oligonucleotides to specific sites on the product. The shorter ss oligonucleotides, also called detection oligonucleotides, will hybridize to the RCA product (18). The detection oligonucleotides have fluorophores conjugated to them, making it possible to detect the protein of interest in a fluorescence microscope (18). The complete *in situ* PLA reaction can be seen in figure 3.

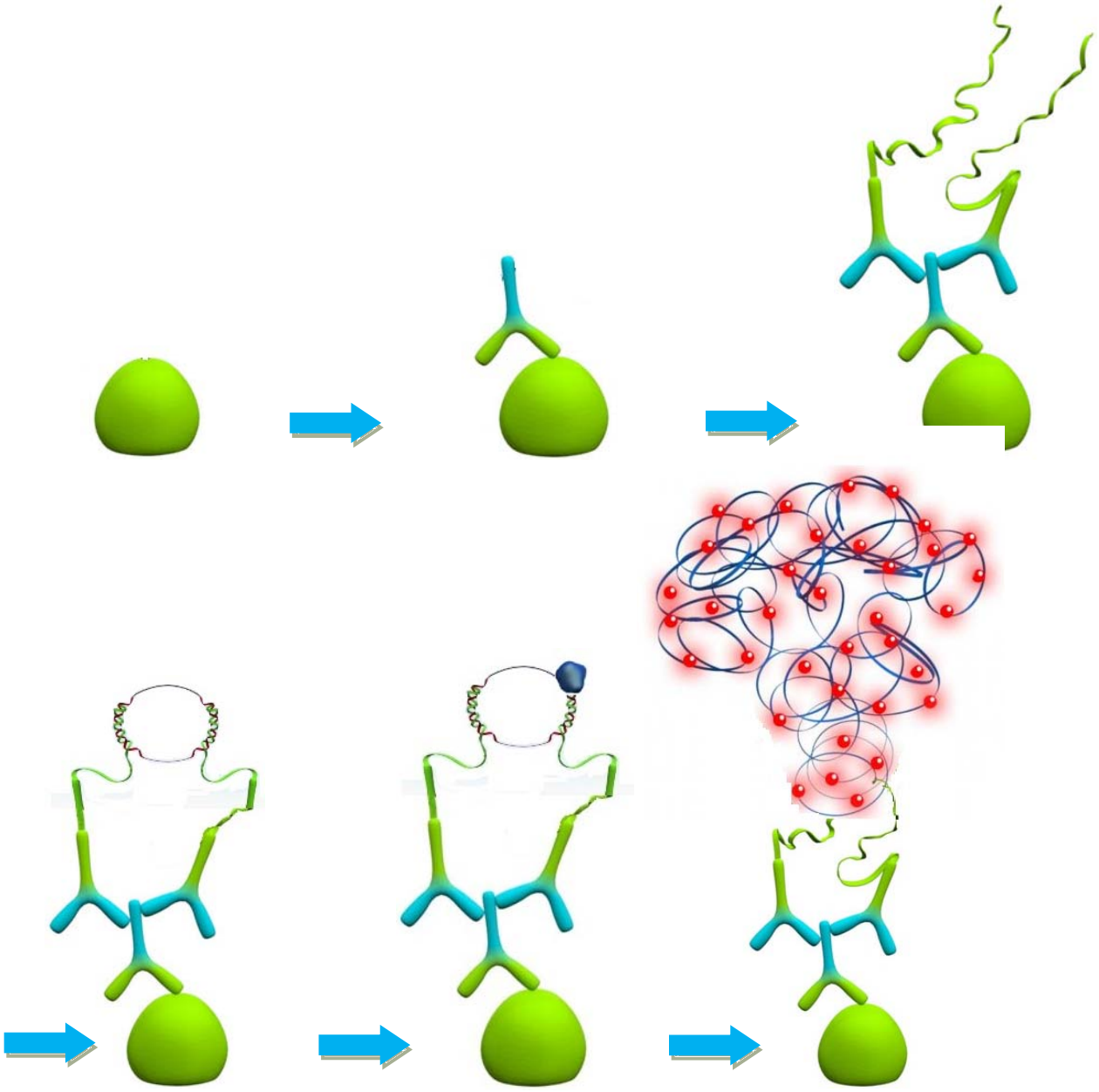


Figure 3. *A schematic description of events taking place in in situ PLA with single recognition. Primary antibody targeting antigen. Proximity probes binds in to bound primary antibody. Connector oligonucleotides hybridize to proximity probes and T4 DNA ligase ligates the single stranded oligonucleotide fragments to a single stranded oligonucleotide circle. Phi29 DNA polymerase amplifies the circle and forming an RCA-product. To the single stranded RCA-product, detection oligonucleotides, with fluorophores conjugated to them, hybridize. Picture used with full permission from Olink Bioscience*

Since *in situ* PLA is utilizing the proximity of two different independent epitopes for the detection of the molecule(s) of interest, a decrease in the probability for false positives occurs. The likelihood for two different proximity probes to bind to two different sites on the molecule(s) of interest is not infinitesimal. However, the probability for the two proximity probes to bind to epitopes in such close vicinity to each other is not high at all. Hence, the likelihood for proximity probes, who have not

bound to their intended target, to result in a signal, is very low. Therefore the probability that a signal does not represent a visualized target is very low when using this method (18).

For every target detection taking place, signal amplification is performed. This increase in signal allows for a better resolution during detection of wanted target. Instead of being able to tell if the target is absent or not, or if the targets exists in larger or minor quantities, *in situ* PLA delivers results of exactly how many targets that was found per cell. This since the RCA product is visible as a fluorescent blob when the sample is studied using a fluorescence microscope (18).

Hence *in situ* PLA can today be used instead of, or as a complement to, IHC and IF when cancer diagnosis is performed on samples such as FFPE tissues. The method will give more quantifiable results, but the issues of normalization standards and the variable ways of fixating tissues remains. By adapting the workflow for the protein detection method so it can be performed on cells in solution, an analysis by flow cytometry would be possible and thereby enabling a more rapid analysis.

2.3.2 Flow cytometry

In order to speed up the analysis of samples, a less time consuming analysing method has to be used. Instead of manually looking at individual cells, flow cytometry offers the possibility to scan up to 10 000 cells per second (19). Despite a high throughput, an individual analysis of every cell is performed. Using scattered light, absorption and fluorescence, individual data for every cell is produced (18).

In a flow cytometer, one cell at a time passes through a laser beam. When doing so, the absorption, forward and side scattered light and fluorescence is measured for every cell, giving information about for example the cells size and shape (20). Combining the flow cytometric analysis with protein detecting methods such as *in situ* PLA, one can detect increases or decreases in protein expression in every individual cell by analysing the eventual increase in fluorescence caused by the detector oligonucleotides hybridized to the RCA product. Since the method allows high throughput screening of larger samples, the analysis of samples, e.g. the search for a few cancerous cells in larger samples, may be not just more effective but also more statistically accurate (21).

In order to analyse samples using flow cytometry, cells must be in solution. Hence this analysing method allows one to avoid the problems concerning FFPE tissues. This analysing method is starting to emerge in cancer diagnostics today (22, 23). Due to the restriction in sample usage, where only cells in solution can be analysed, cancer diseases such as leukaemia and lymphomas are being diagnosed today (23). By combining flow cytometry with *in situ* PLA, the combined methods allows for a more safe, accurate and fast diagnosis of cancer diseases such as leukaemia and lymphomas due to the lowered risk for cross reactivity and high throughput analysis.

2.4 The need for improved diagnostic tools

Today, invasive methods are being used for the diagnosis of cancer. Biopsies may have to be performed in order to establish an exact diagnosis, an operation that might be discomforting for the patients as well as exposing them to certain risks such as the risk of infections. Also, fixation of tissues may be performed in different ways at different laboratories, meaning that results cannot be compared between laboratories. On top of this, the diagnosis itself is a questionable process. The procedure of diagnosing cancer demands experienced pathologists rather than well performed

laboratory work, since methods such as IF and IHC do not give quantifiable results. Thus, improvements in the diagnosis of cancer are of interest for all parties involved.

A less dramatic and dangerous procedure for the collection of sample from patients would be preferred over biopsies. A less invasive technique, such as fine needle aspiration biopsy (FNAB), would ease the patients discomfort as well as lower the risk for complications. FNAB is under development today, and may, in a near future, be used for sample collection with the purpose to diagnose cancer (24). Furthermore, this new method for sample collection delivers smaller and more manageable samples, which opens up for new ways of diagnosis. By escaping the requirements of tissues for morphological and protein expression studies, one will also avoid the issues concerning sample preparation. Reducing the need to fixate tissues and cells for diagnosis, opens up for the usage of new methods and with that also lowers the demand of experienced pathologists. This, in turn, will enable diagnosis in large scale. Reducing sample preparation, and thereby the amount of work put into the diagnosis, will speed up the process and also reduce the possibilities for human errors. Also, opportunities to easier diagnose certain cancers only by the investigation of protein expression opens up. More quantifiable data resulting from non-fixated cells or FFPE tissue samples will be easier to interpret. Hence, it would be possible to see, in a more exact manner, how much the expression of certain proteins is up or down regulated.

Analysing tissues today takes time. Apart from the laboratory work, pathologists must study the tissues very carefully in the search for carcinogenic cells. This analysing method is time consuming and does not enable larger scale analysis. Using *in situ* PLA combined with flow cytometry, not dependent on samples such as FFPE tissues, allows for a rapid and exact analysis of samples. This combination of methods may also enable diagnosis of cancers where only a few cancerous cells can be detected in a larger sample. Hence, this new combination of methods may allow for detection of only a few circulating tumour cells (ctc's) in blood samples. However, this combination of methods does not exist today.

2.5 Description and aim of the project

This project aims to develop new reagents and workflows for improved performance of *in situ* PLA with flow cytometric readout. In order to analyse samples that has been subjected to *in situ* PLA using flow cytometry, the *in situ* PLA method must be able to be performed on cells in solution. The method must also provide sufficient signals that can be detected with flow cytometry analysis.

2.6 Problem solving

In order to be able to combine flow cytometry with *in situ* PLA, a new workflow for *in situ* PLA must be developed where the reaction can be performed on fixed cells in solution. Since it has been reported that cell retrieval is a big issue when trying to perform *in situ* PLA on cells in solution (20), different separation methods will be investigated. Here, the main focus will concern cell retrieval after performed *in situ* PLA protocol.

Also, the developed *in situ* PLA method must provide a signals strength that can be detected using flow cytometry. Here the project aims to maximize the PLA-signals within the cells by investigating the impact of permeabilization of cell membrane and reagent concentrations. In addition, an attempt to increase the total fluorescence of the PLA-signals is performed in order to investigate the sensitivity of the analyzing method.

3 Results

Throughout this degree project the possibility to develop a method, based on the *in situ* PLA technique combined with flow cytometry, for the diagnosis of various cancer diseases was investigated. The existing *in situ* PLA technique was applied to cells in solution and optimisations of the protocol was performed for the development of a new, more sensitive and selective, diagnostic method where flow cytometry enables high throughput analysis.

3.1 Resulting signals and cell retrieval

When producing a diagnostic method that performs an individual analysis of every cell, it is of great interest to maintain as many cells as possible throughout performed *in situ* PLA reaction. Separation methods used for separation between cells and surrounding solution is a bottleneck concerning cell retrieval. Three types of separation methods were investigated. One separation method involving magnetic particles with bound antibodies, having the task to capture cells by exploiting specific epitopes on the outer cell membrane, were examined. This method was not to be of primary choice due to the selective separation of cells from surrounding solution. Results showed a poor cell retrieval (data not shown), and therefore we focused on the remaining two separation techniques, filtration and centrifugation.

When comparing the effectiveness of the filtration and the centrifugation technique, the amount of PLA-signals per cell are of importance, as well as the cell retrieval. It is desired to achieve a workflow were as few cells as possible are lost. This due to the possibility that only a small portion of the investigated sample may contain carcinogenic cells and the chance to lose those few cells during performed protocol should be as low as possible. Also the signal strength is of importance since a few carcinogenic cells should be able to be detected in a solution also containing a large number of healthy cells. Earlier attempts to perform *in situ* PLA on fixed cells free in solution had used centrifugation as a separation method with poor cell retrieval as a result (21). This was also the case here, when using same centrifugation speed and time (data not shown). Therefore optimisations concerning centrifugation speed, centrifugation time and amount of cells in starting material was performed (for complete procedure, see material and methods section 5.2.1), raising the cell retrieval substantially.

When *in situ* PLA was performed on cells in solution and filtration, or centrifugation, was used to separate the cells from reagents, it was shown that both methods effectively maintained a larger portion of the cells present in the starting material. A summary of the effectiveness of the separation methods can be seen in figure 4. Reference represents amount of cells in starting material.

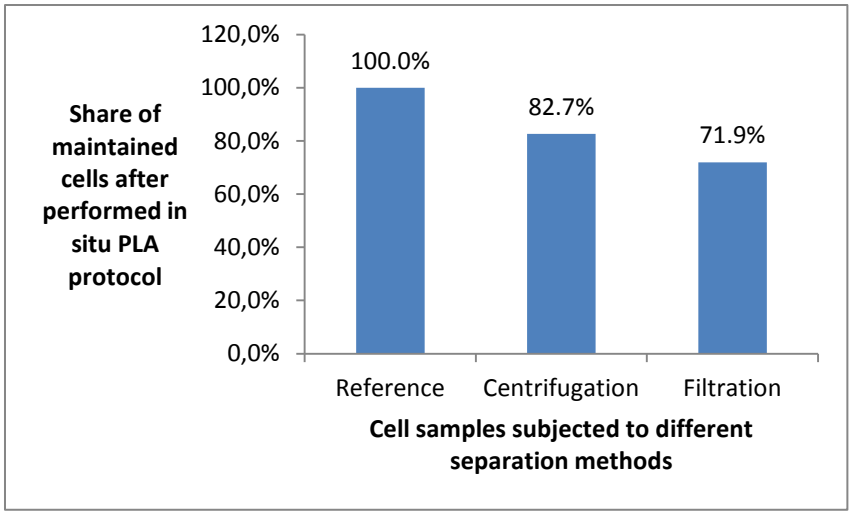
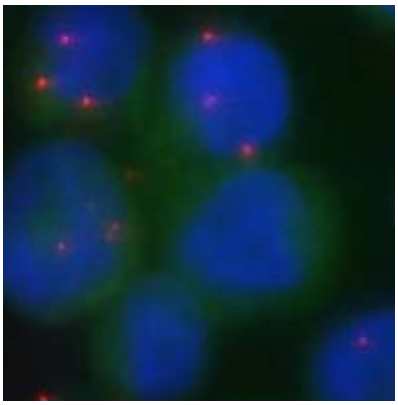


Figure 4. *An overview of the effectiveness of the filtration and centrifugation techniques measured in amount of remaining cells after performed in situ PLA protocol. Both separation methods, centrifugation and filtration, gave rise to a high cell retrieval. Here the reference used is the starting material.*

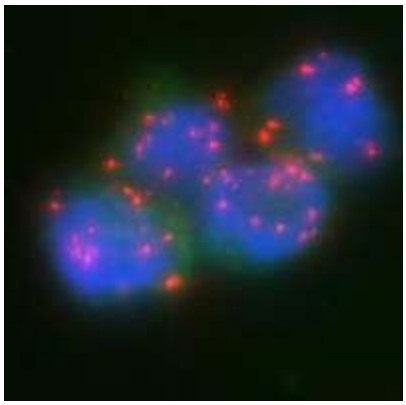
It can clearly be seen from figure 4 that no big loss in cell number is being noted regardless of chosen separation method. Hence the effectiveness concerning cell retrieval is high for both techniques.

Although the cell retrieval has been proven to be promising for both filtration and centrifugation, an analysis of the resulting PLA-signals demonstrates an increase in amount of signals for filtrated cells. As can be seen from figure 5, the amount of resulting PLA-signals is greater for the filtrated cells compared to the centrifuged cells.

A



B



C

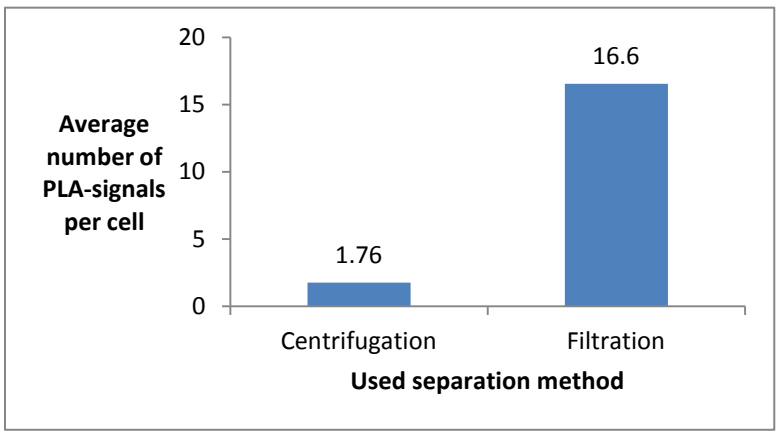


Figure 5. *Resulting PLA-signals when performing in situ PLA on cells in solution where centrifugation or filtration technique has been used as separation methods.*

A) Resulting PLA-signals in centrifuged cells. Picture taken using a fluorescence microscope. Staining of cell nucleus using DAPI gives blue nuclei. PLA-signals can be seen as red dots. Green colouring results from FITC-marked primary antibody.

B) Resulting PLA-signals in filtrated cells. Picture taken using a fluorescence microscope. Staining of cell nucleus using DAPI gives blue nuclei. PLA-signals can be seen as red dots. Green colouring results from FITC-marked primary antibody.

C) The average number of PLA-signals in filtrated cells is greater than for centrifuged cells. Filtered cells gave rise to more PLA-signals per cell

This observation is most certainly linked to the restrictions in minimum amount of cells in the starting material when using centrifugation as a separation method. In order to form clearly visible pellets when centrifuging, the starting material must contain a large amount of cells compared to when using filtration as a separation method. This constrains the user to a larger reaction volume. However, the reaction tubes used limit the amount that can be added to the pellet. When adapting the reaction volumes, and hence amount of reagents added to every cell in the sample, one could see a more even distribution of PLA-signals per cell between centrifuged and filtered cells (representative data shown in section 3.4).

The number of signals per cell plays an important role when using flow cytometry as an analysing method. As can be seen from figure 6, a large deviation between results from the filtered and centrifuged cells can be noticed when studying the samples.

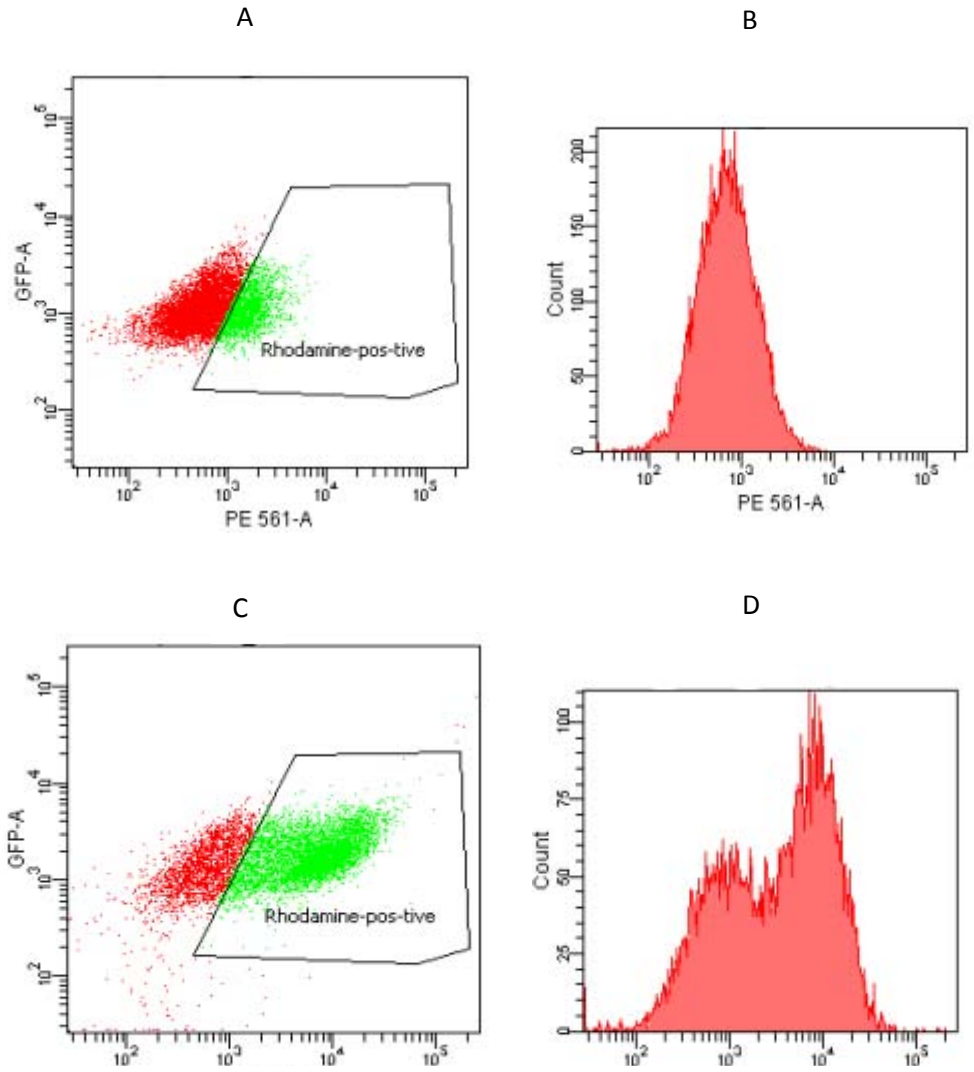


Figure 6. *Resulting data from flow cytometric analysis of samples where in situ PLA has been performed on cells in solution. Figure 6 A and B display flow cytometric results when centrifugation has been used as a separation method, and C and D demonstrate results where filtration has been used to separate cells from surrounding solution. Rhodamine fluorescence indicates PLA-signal and GFP fluorescence displays the presence of primary antibody.*

A and C) Overview of GFP and Rhodamine fluorescence for investigated cells in sample. Every dot represents the total GFP and Rhodamine fluorescence for one cell. The fluorescent rhodamine intensity can be seen on the x-axis, and the fluorescent GFP intensity on the y-axis. PLA-signals in cells increase the rhodamine intensity.

B and D) Summary of the mean Rhodamine fluorescence for the whole cell population in the sample. Fluorescent rhodamine intensitycan be seen on the x-axis and number of cells on the y-axis.

As can be seen from figure 6 B and 6 D, more signals per cell are observed as an increase in the total mean PLA-signal fluorescence when analyzing the sample with flow cytometry. Since the total amount of signals in cells will increase the rhodamine fluorescence in the cell, as well as the mean rhodamine fluorescence in the cell population, an increase in PLA-signals will affect the mean PLA-signal fluorescence when analyzing the sample with flow cytometry. Two peaks can be noted in figure 6 D, suggesting that more PLA-signals increase the total fluorescence in the cells and thereby also makes them easier to distinguish from cells that lack PLA-signals. Furthermore, the increase in signals gives a higher rate of rhodamine positives, figures 6 A and 6 C, which indicates cells that has been subjected to *in situ* PLA.

Thus, the filtration technique is as effective as the centrifugation technique when it comes to cell retrieval. Due to the need of larger starting material when using centrifugation as a separation technique, and thereby also larger reaction volumes, the centrifugation technique can advantageously be used on larger samples.

Restrictions in amount of cells in starting material also exist for the filtration technique. Larger samples, hence larger amount of investigated cells, may clog the pores in the filter and therefore not allow drainage of solutions. Investigation of how large samples that could be studied when using filtration as a separation technique, showed a clear limitation in the amount of cells investigated.

Table 1. *Investigation of amount of cells that can be used in starting material without complications when separating cells from surrounding solution*

Filtration tubes	Number of cells	Total volume before drainage (µl)	Remaining solution (µl)
Sample 1	80 000	500	0
Sample 2	160 000	500	0
Sample 3	240 000	500	<20
Sample 4	320 000	500	20
Sample 5	400 000	500	<30
Sample 6	480 000	500	30
Sample 7	560 000	500	<40
Sample 8	640 000	500	40
Sample 9	720 000	500	<70
Sample 10	800 000	500	<80

Remaining volume of solution after separation should be as small as possible. Table 1 shows that by using starting materials containing 320 000 cells or less, pores in used filter does not clog and solution can be drained effectively.

Table 1 shows that by adding more than 320 000 cells to one filtration tube, one will have trouble to separate solutions from the cells when centrifuging for 500 x g for 1 minute. Therefore, for samples larger than 320 000 cells centrifugation can be used as a separation technique, or the centrifugation time when filtering may have to be prolonged. Due to the filters ability to detain solution, and therefore maintain moisture, residual volume can advantageously be as small as possible.

When centrifuging larger samples the amount of cells in the starting material have an impact on the pellet formation. In order to reduce the loss of cells throughout performed *in situ* PLA protocol, the

structure and visibility of the cell pellet is of importance in order to allow for an easy pipetting of the supernatant. A number of samples (table 2), containing different amounts of cells in their starting material, were centrifuged once. When investigating minimum amount of cells in the starting material one could note that about 444 000 cells were needed in order to form a visible pellet.

***Table 2**. Investigation of minimum amount of cells that can be used in starting material to form rigid pellet.*

Sample	Added 1 x PBS (µl)	Cell sample (µl)	Number of cells in starting material
Sample 1	980	20	148 000
Sample 2	970	30	222 000
Sample 3	960	40	296 000
Sample 4	950	50	370 000
Sample 5	940	60	444 000
Sample 6	930	70	518 000
Sample 7	920	80	592 000
Sample 8	910	90	666 000
Sample 9	900	100	740 000
Sample 10	890	110	814 000
Sample 11	880	120	888 000
Sample 12	870	130	962 000
Sample 13	860	140	1 036 000
Sample 14	850	150	1 110 000
Sample 15	840	160	1 184 000

Overview of centrifuged samples, containing different amounts of cells in their starting material. Visualization of pellet was controlled for all samples. In samples containing 444 000 cells or more in their starting material, cell pellets were detected.

Apart from the visual control of pellet formation, the cell retrieval after the centrifugation was investigated. The average number of cells per picture taken, using a fluorescence microscope, (figure 7) clearly showed an increase in cell retrieval at approximately 370 000 – 445 000 cells.

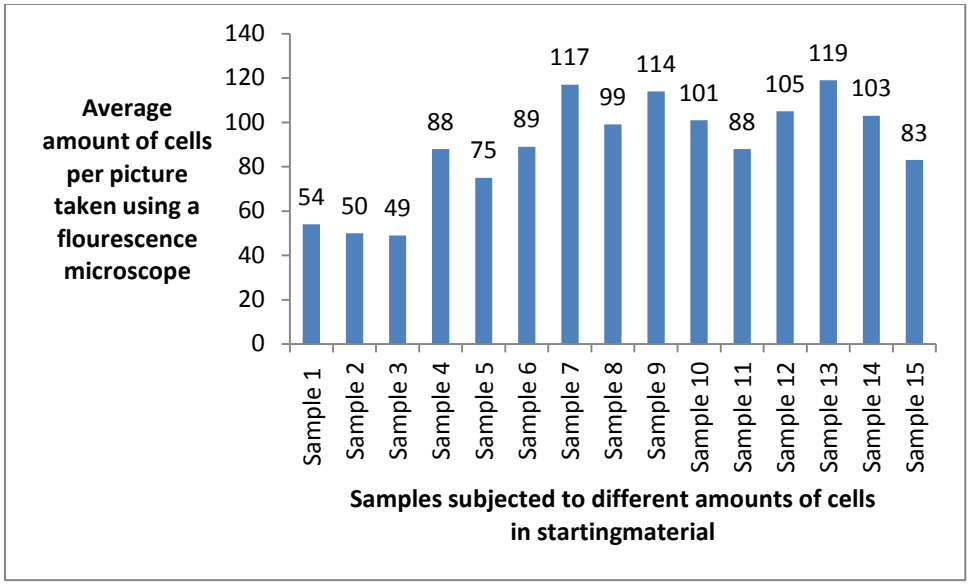


***Figure 7.** Overview of average number of retrieved cells after centrifugation of samples containing different amounts of cells in the starting material. Samples containing starting material with 370 000 cells or more gave higher cell retrieval.*

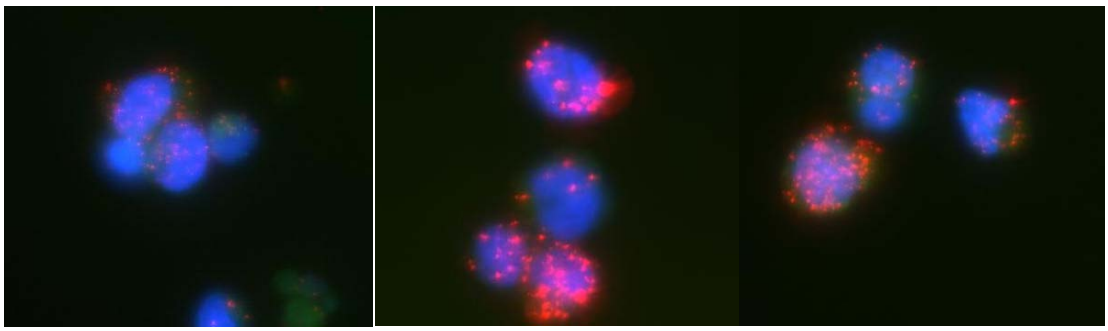
Thus, the results suggests that for maximum cell retrieval, when using centrifugation as a separation method, samples containing about 370 000 cells or more can be used. A visual pellet eases the pipetting of the supernatant and hence increases amount of maintained cells in sample.

3.2 Optimisation of protocol

The developed diagnostic method should have as high precision as possible when discriminating between healthy and cancerous cells. This is accomplished by lowering the number of false positives, allowing the true positive signals to easier be detected. By increasing the amount of resulting PLA-signals in cells and / or increase the total fluorescence intensity of the existing signals, a more robust and sensitive analysis using flow cytometry is possible. To achieve this, an optimisation of current protocol was performed.

3.2.1 Amount of signals per cell relates to permeabilization

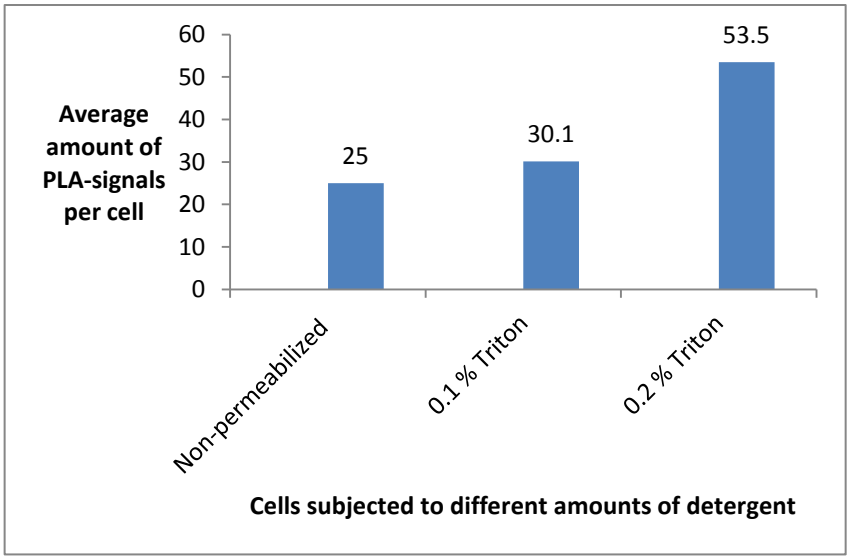
When using antibodies that are targeting antigens located inside cells, in this case a primary antibody targeting actin, permeabilization of the cell membrane may be of importance. In order to investigate what concentration of the detergent Triton that efficiently will permeabilize, without breaking the cells, a study of the resulting PLA-signals in cells was performed. Here, cells permeabilized with 0.1% and 0.2% Triton, as well as non-permeabilized cells, were investigated.



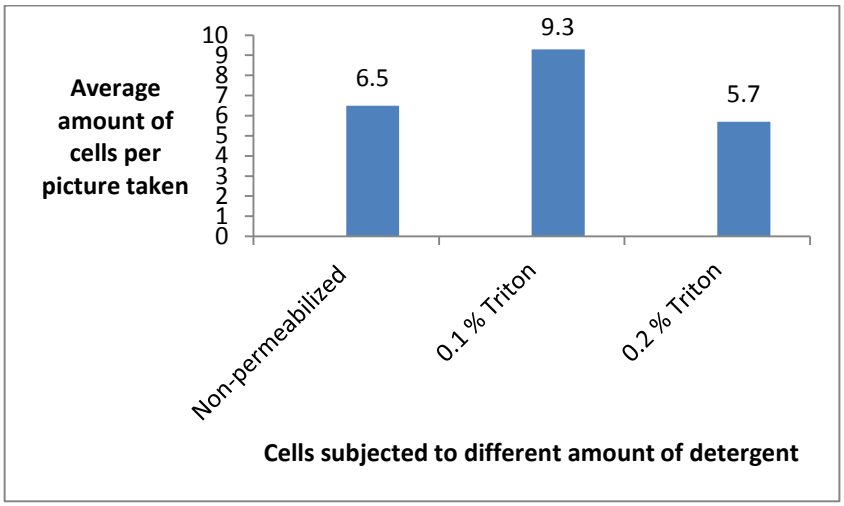
A B C

Figure 8. *Images of resulting PLA-signals when performing in situ PLA on cells in solution where permeabilization of cell membrane has been performed using different concentrations of Triton. Staining of cell nucleus using DAPI gives blue nuclei. PLA-signals can be seen as red dots. An increase in PLA-signals per cell can be noted when increasing the concentration of the detergent.*
A) Resulting PLA-signals in cells that has not been permeabilized.
B) Resulting PLA-signals in cells permeabilized with 0.1 % triton.
C) Resulting PLA-signals in cells permeabilized with 0.2 % Triton.

As can be seen from the resulting images in figure 8, a big difference in amount of signals can be detected between the permeabilized and the non-permeabilized cells. Hence, the performed disruption in the cell membrane allows for antibodies to better penetrate cells, and thereby more easily reach their target antigen. From figure 9A, one can see the average number of PLA-signals per cell in one picture taken using a fluorescence microscope. This diagram shows that the amount of resulting signals increases with increased concentration of Triton. Figure 9B displays the average number of cells per picture, and reveals that no larger cell loss can be detected when increasing the concentration of detergent.



A



B

Figure 9. *A summary of cell retrieval, and PLA-signals per cell, when using different concentrations of detergent. A) An overview of the resulting PLA-signals per cell, when in situ PLA is performed on cells subjected to different permeabilization techniques. A concentration of 0.2% Trition gave rise to a high number of PLA-signals per cell. B) A summary of the average cell numbers per picture taken using a fluorescence microscope. A high concentration of detergent, 0.2% Trition, did not negatively affect the cell retrieval.*

Hence, a concentration of 0.2 % Triton can advantageously be used in order to increase the number of antibody – antigen interactions without destroying a larger part of the sample.

3.2.2 Amount of reagents affects resulting signals

In order to more easily distinguish cancerous cells from healthy cells, the amount of resulting PLA-signals should be as high as possible for the true positives. *In situ* PLA-reagents should be added in such amounts that all present proteins of interest, within or on the membrane of all cells, should be able to be detected. Hence, in order for RCA products to form and for detection oligonucleotides to attach to the RCA products, enough reagents must be added to the cell mixture. In order to find the minimum amount of reagents that give rise to a maximum amount of PLA-signals, a series of different reaction volumes, 100, 150, 200, 250 and 300 µl, were added to a fixed number, 240 000 , of cells. During this trial, the concentrations of all reagents were fixed.

When investigating the resulting PLA-signals in the different samples, one could clearly see that a maximum value of PLA-signals can be reached with a reaction volume of 200 µl, and that even larger reaction volumes not will be beneficial for increased amount of signals. As can be seen from figure 10, a reaction volume of 200 µl gives the highest average amount of resulting PLA-signals per cell, and reaction volumes above 200 µl seems to decrease the amount of detected signals. One can also see that there is a big difference in resulting PLA-signals when using 100 and 150 µl reaction volumes, indicating that one or more reagents restricts the signal production.

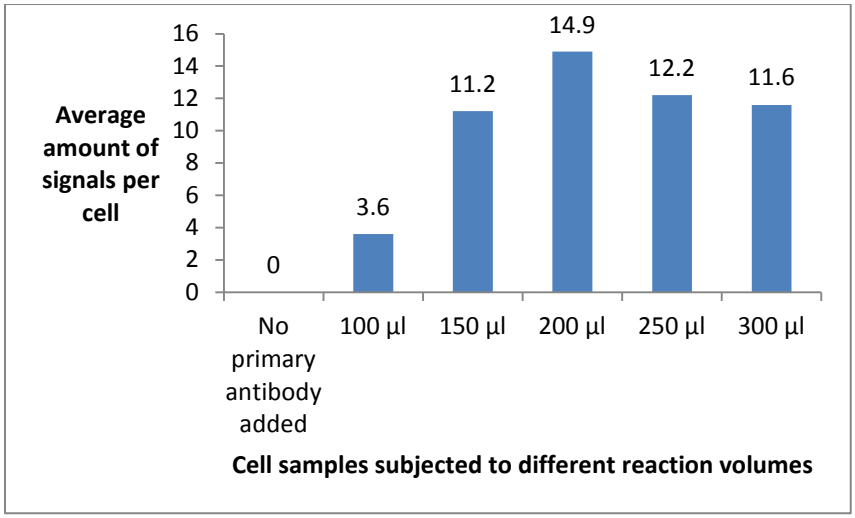


Figure 10. *Overview of the average resulting PLA-signals per cell when investigating how different reaction volumes, added to 240 000 cells, affects the resulting amount of signals. Here reaction volumes of 100, 150, 200, 250 and 300 µl have been added to a fixed amount of 240 000 cells. A reaction volume of 200 µl gave rise to largest amount of PLA-signals per cell.*

Hence, by using reaction volumes of 200 µl for every 240 000 cells, one can maximize resulting amount of PLA-signals. This specific value also allows for scaling trials up and down.

3.2.3 Amplification time affects the intensity of the PLA-signals

When using flow cytometry as an analysing method, the intensity of the PLA-signal is of importance. Since the total mean fluorescence of each registered cell is measured, every fluorophore within the cell contributes to the recorded signal. By increasing the amount of detection oligonucleotides that can bind in to an RCA product, the intensity of every PLA-signal increases, and hence it may be easier to distinguish cells with PLA-signals from cells that lack signals.

When investigating the correlation between amplification time and intensity of resulting PLA-signals using flow cytometry, one could clearly see a connection. By amplifying the RCA product for a longer period of time, the total mean rhodamine fluorescence increased. As can be seen from figure 11, longer amplification times gave rise to higher total mean fluorescence for the cell population, and hence a relocation of the peak to the right in the resulting histogram. When comparing the resulting fluorescence from six different amplification times (figure 11 A – G) one can clearly see that the peak moves to the right, corresponding to an increase in fluorescence for all cells in the investigated population.

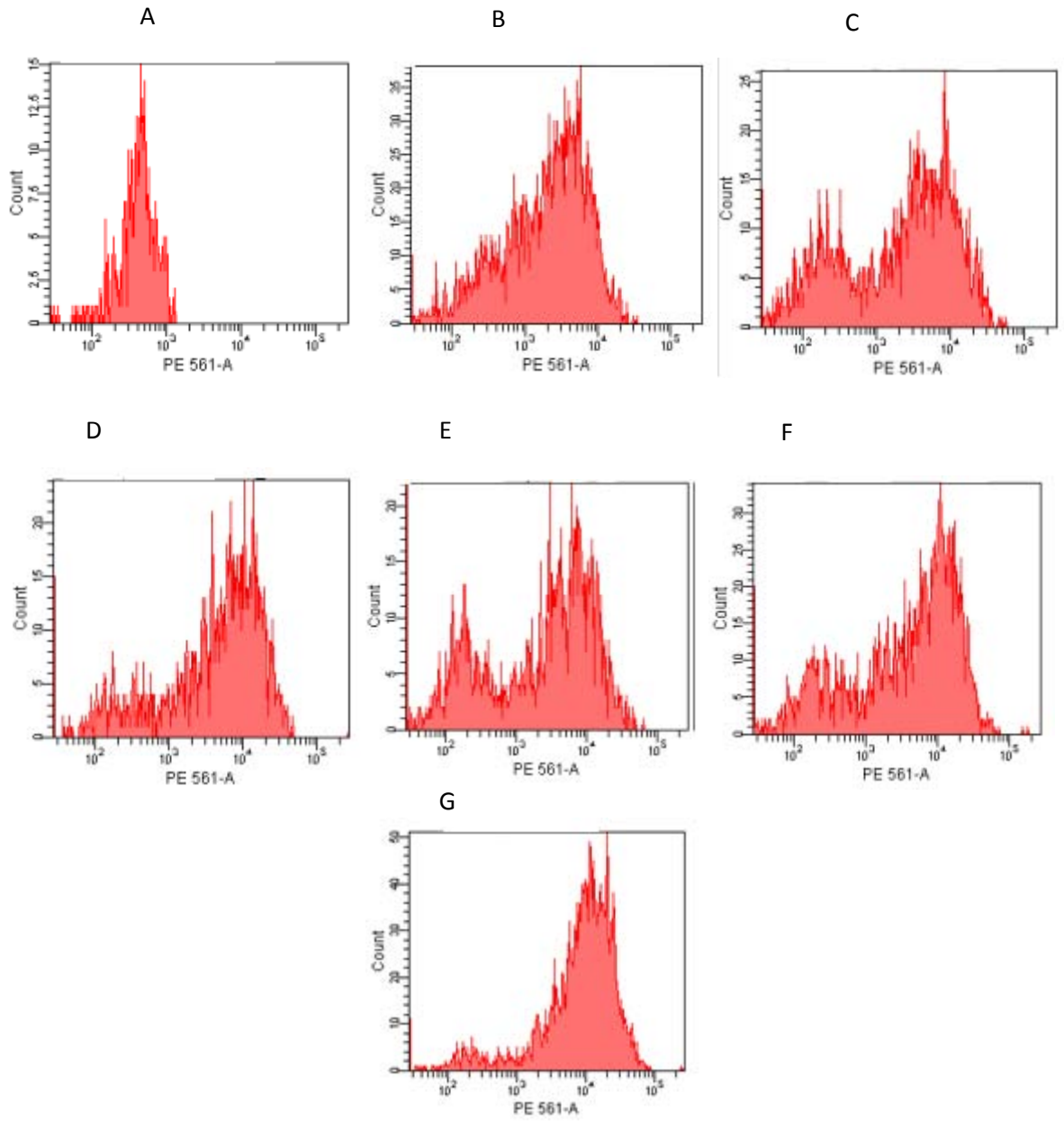


Figure 11. *Overview of the total mean rhodamine fluorescence in cells when increasing the amplification time when performing in situ PLA. Analysis is performed using flow cytometry on samples where in situ PLA has been performed on cells in solution. Filtration has been used as separation technique. The fluorescent rhodamine intensity can be seen on the x-axis and number of cells on the y-axis. Longer amplification time results in more intense rhodamine fluorescence. Amplification times exceeding 135 minutes separates cells containint PLAsignals from those that does not.*

A) Overview of the total mean rhodamine fluorescence of cells where no primary antibody has been added. Here the RCA product has been amplified for 100 minutes.

B – G) Overview of the total mean rhodamine fluorescence of cells subjected to in situ PLA reaction. In figure B the RCA product has been amplified for 100 minutes, in figure C for 135 minutes, in figure D for 165 minutes, in figure E for 195 minutes, in figure F for 225 minutes and in figure G for 255 minutes.

Something that also can be noted is that another peak is appearing in the diagram when amplifying the RCA product for 135 minutes or more. The peak to the left represent cells containing no PLA-signals and the peak to the right symbolize cells containing PLA-signals. By increasing the amplification time of the RCA product, the peak representing the true positives shifts further away from the peak representing the true negatives. By doing so, we are able to easier distinguish between cells with and without PLA-signals all present in one complex sample.

3.3 Emitted light from fluorophores affects the sensitivity of the analysis

Since a flow cytometer measures the total fluorescent intensity of cells at certain wavelengths, the choice of fluorophore, linked to the detection oligonucleotides, might affect the sensitivity of the analysis. Dependent on the intensity of the emitted light derived from used fluorophores, PLA-signals may be perceived as more or less intense and hence result in a more or less sensitive diagnostic method. To investigate the possible effects that different fluorophores may have on the flow cytometric analysis, a dilution series of the primary antibody was performed in order to obtain a double set of cell populations with decreasing amounts of PLA-signals. To one set of the series the fluorophore Far Red was used, and to the other set Orange was added.

The results clearly stated that different fluorophores do affect the sensitivity of the flow cytometric analysis. However, due to the possibilities to set different exposure times when analyzing cells using a fluorescent microscope, the drawbacks with usage of less intense fluorophores can be compensated for.

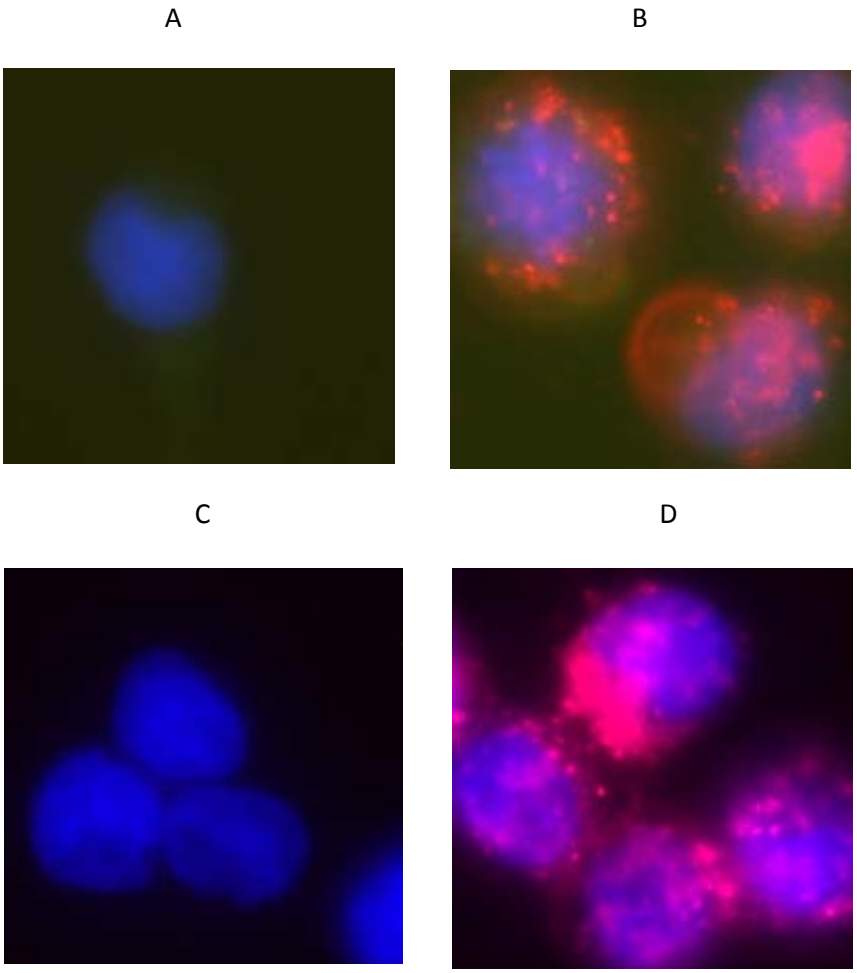


Figure 12. *Comparison of PLA-signals when the flourophores Far Red or Orange are used.*

A and C) Cells not subjected to primary antibodies. Orange connected to detection oligonucleotide was used in A and Far Red was used in C. No PLA-signals are expected, and none could be seen.

B and D) Cells subjected to complete in situ PLA reaction. In B, Orange was the used fluorophore and in D Far Red. Approximately equal amounts of signals per cell could be seen regardless of fluorophore used.

As can be seen from figure 12, the amount of resulting PLA-signals is approxemately the same regardless of type of fluorophore used, when analysing cell samples with a fluorescence microscope. This is achieved by applying different exposure times, for Orange 300 ms and for Far Red 5000 ms, on the samples. By increasing the exposuretime for the not so intense fluorophore Far Red, one can enhance the visible signale and hence just as easily detect PLA-signals when Far Red is used as when Orange is used. More data can be seen in suplementary data 1S.

Since the exposure time for the cell samples analysed by flow cytometry is not adapted according to the intensity of emitted light from used fluorophores, a greater difference in fluorescent intensity between samples containing the different fluorophores can be seen in figure 13. Despite the fact that cells are almost saturated with PLA-signals, flow cytometric analysis displays a large number of cells registrated as non-holders of PLA-signal when Far Red was used as fluorophore. Furthermore a wide distribution of registrated intensities can be seen, and hence the resulting peak in the histogram is not as clearly defined as for the one registrated when Orange was used as fluorophore.

Due to the large number of false negatives that occur when using Far Red, the sensitivity of the analysing method is strongly reduced. As can be seen from supplementary data 1S and 2S it is possible to distinguish cells containing two PLA-signals from those that contain non, when using orange as chosen fluorophore. However, when using Far Red the possibility to distinguish cells with low amounts of PLA-signals from cells without signals becomes considerably more difficult. This is due to the massively increased amount of false negatives that is registrated.

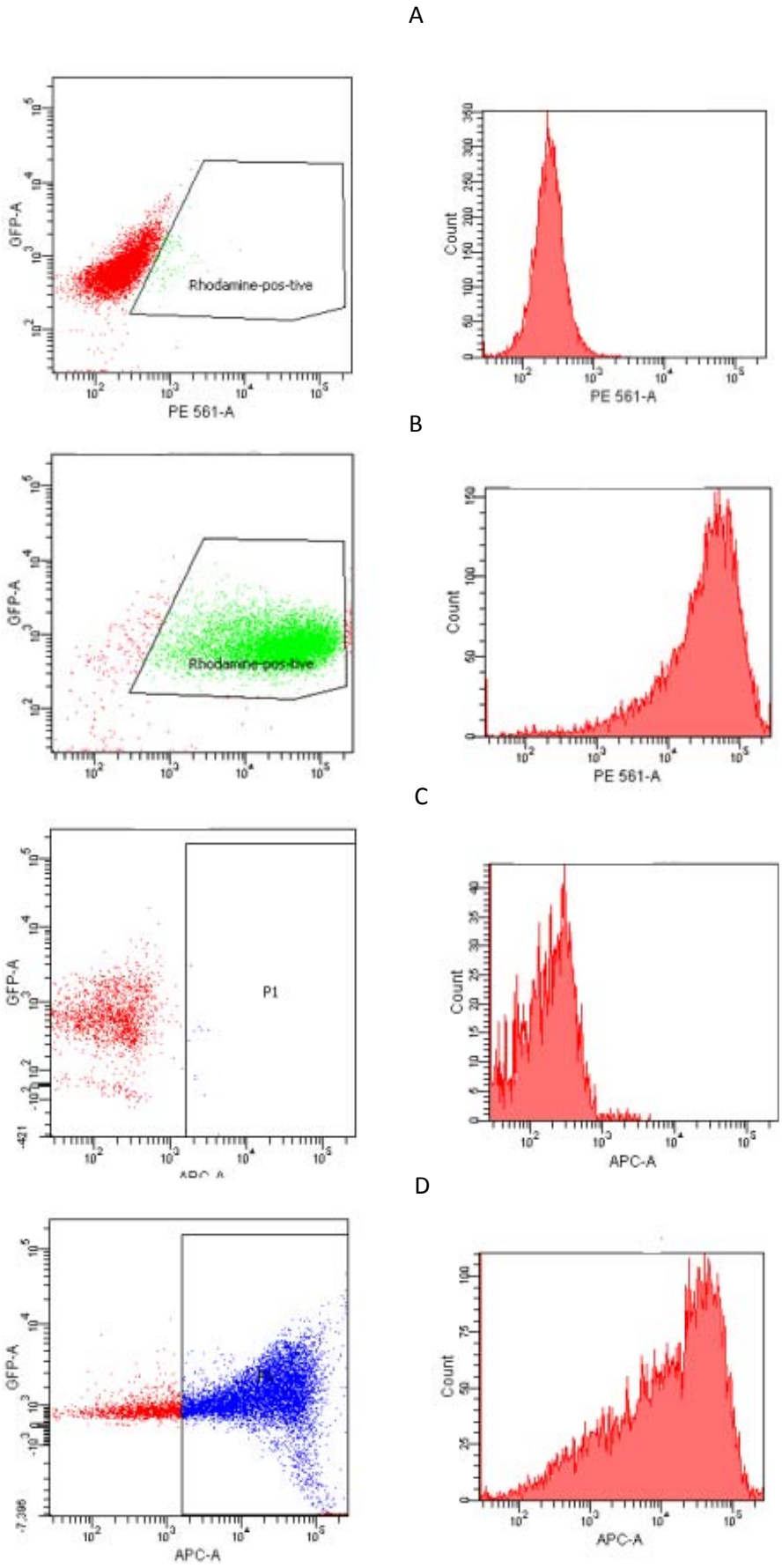


Figure 13. *Overview of amount of cells registered as true positives, defined as signals registered in areas named Rhodamine-positives and P1, can be seen in left diagram for figure A – D. Here the fluorescent intensity of used fluorophore can be seen on the x-axis, and the GFP fluorescens can be seen on the y-axis. Diagrams to the right represent the total mean fluorescence of specified fluorophore for the cell population. Here the fluorescent intensity of used fluorophore can be seen on the x-axis, and number of cells on the y-axis.*

A and B) Here Orange were the used fluorophore. In figure A, no primary antibody has been added to the cells and no PLA-signals are hence present. In figure B cells are almost saturated with PLA-signals, and almost all cells are registered as true positives.

C and D) Here Far Red was the used fluorophore. In figure A, no primary antibody has been added to the cells and no PLA-signals are hence present. In figure B, cells are almost saturated with PLA-signals, but only a few cells are registered as true positives. Hence, by using the fluorophore Far Red many cells are registered as false negatives.

3.4 Comparison of assay performance

In order to get an idea of the assay performance when performing *in situ* PLA on cells in solution in relation to when performed the conventional way, on cells fixated onto glass slides, a comparison between the methods were made. Cytospin, containing the same cells as the solution, was used for the performance of *in situ* PLA on cells fixated onto glass slide. For more information about performance of *in situ* PLA on the different samples, see materials and methods, section 4. This comparison primarily focused on the average amount of PLA-signals per cell as well as amount of signals on glass slides, here called the background.

As can be seen from figure 14 the average amount of PLA-signals per cell is of the same magnitude, regardless if the *in situ* PLA is performed on cells in solution or on cells fixated onto glass slide. However, when studying the background, that is the false positive signals appearing on the glass slide outside cells, one can see a significant increase when the *in situ* PLA method is performed on cells fixated onto glass slides. This result therefore suggests that the efficiency of the method, and hence the assay performance, is better when performing *in situ* PLA on cells in solution.

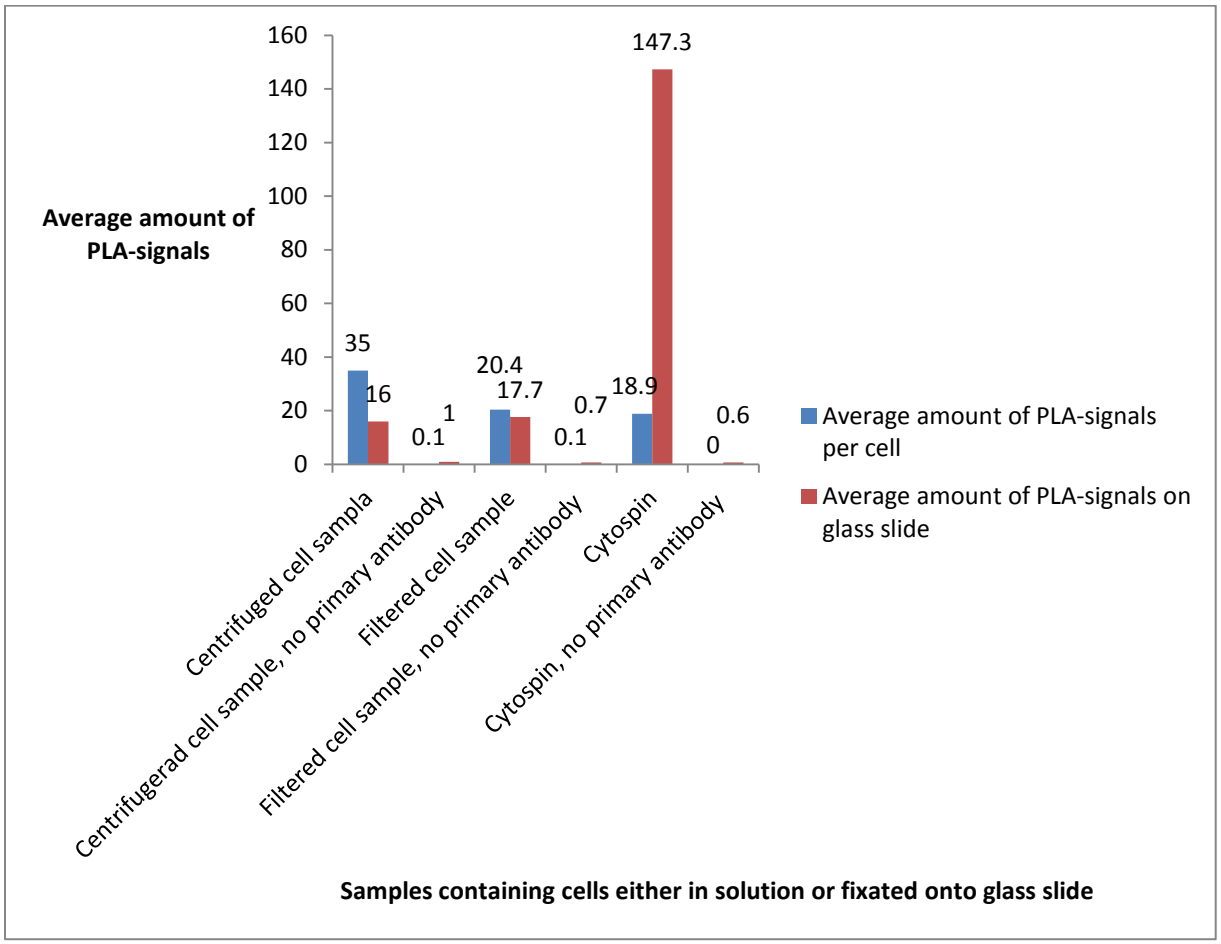


Figure 14. *Overview of average amount of PLA-signals within cells free in solution and in cells fixated onto glass slides, as well as amount of signals outside cells. More signals per cells is achieved by performing in situ PLA on cells in solution and using centrifugation as a separation method. Amount of signals outside cells, thus signals on glass slide, is much higher when performing in situ PLA on cells fixed onto glass slides.*

3.5 Performing *in situ* PLA on cells in solution using optimized workflow

By combining all protocol optimisations described above, the new workflow result in a more sensitive protein detection method, and thereby a more sensitive diagnostic tool. In order to investigate the possible enhancement in sensitivity, different samples of cells containing different amounts of PLA-signals were analysed using flow cytometry. In order to produce different amounts of PLA-signals in cell samples, a dilution series of the primary antibody was used (complete data shown in supplementary data 3S). When analyzing the samples in a fluorescent microscope, the average number of signals per cell was estimated, see figure 15. One could note that not all cells contained PLA-signals when a low concentration of primary antibody, 6 – 0.75µg/ml, was used. Another thing that also was registered was that the sample exposed to12 µg/ml of the primary antibody contained such large amount of PLA-signals that the cells appeared saturated with signals. This state strongly affects the resulting analysis when using a fluorescent microscope. The software used is not able to distinguish separate signals and hence the total amount of signals is not correctly detected in the analysis. The estimated number of signals in cells exposed to 12µg/ml of primary antibody is therefore higher than shown by data.

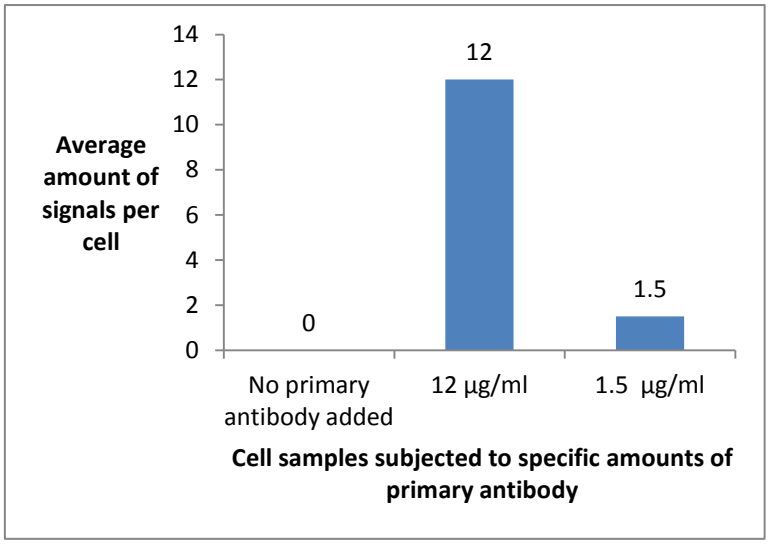


Figure 15. *An overview of the average number of PLA-signals per cell when in situ PLA was performed on cells in suspension. A larger amount of primary antibody gives rise to larger amounts of PLA-signals per cell.*

As can be seen from figure 15, a reasonably low amount of PLA-signals can be found in cells subjected to a low concentration of primary antibody. When analyzing all samples using flow cytometry, an estimate of the method's sensitivity was able to be established (all data shown in supplementary data 4S). As is shown in figure 16, the sensitivity of the method is high. As previously noted, samples with low concentrations of primary antibody contain cells that are lacking PLA-signals. This can be seen in figure 16 C, where the histograms clearly display two peaks. The first peak, having the lowest rhodamine intensity, is consistent with the negative control, indicating that intensity of cells displayed here are cells that are lacking PLA-signals. Peak number two indicate cells with increased rhodamine intensity, and hence cells containing PLA-signals.

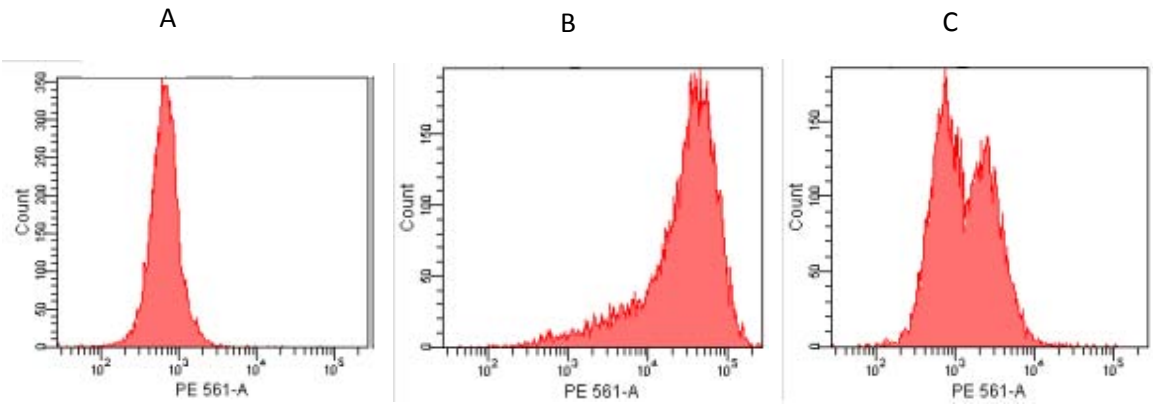


Figure 16. *Overview of resulting total mean rhodamine intensity when performing in situ PLA, using optimized protocol, on cells in solution. Number of cells can be seen on the y-axis and total mean fluorescent intensity of the cell population on the x-axis.*

A) Figure 10A indicates a negative control where no primary antibody has been used.
B and C) Here a final concentration of 12 µg/ml and 1.5 µg/ml of primary antibody has been used. The sensitivity of the analysis is high since a distinction between two peaks can be seen in figure 10C. Hence, the analyzing method can distinguish between cells with on average 1.5 PLA-signals per cell from cells containing no signals.

The results show that it is possible to detect cells containing roughly 1.5 PLA-signals in average from cells that are lacking signals. From those results one can conclude that the developed method has a high sensitivity and that it has the ability to detect minor changes in protein expression.

3.6 Problem arises when deviating from requirement of probes in proximity

It is known that the requirement of binding two PLA-probes in close proximity, in order to form an RCA product and hence a PLA-signal, reduces the impact that cross reactivity has on the analysis. By lowering the amount of false positives, in situ PLA is a more specific and sensitive protein detection method compared to e.g. IHC and IF. In order to specify the need for low numbers of false positives for an increased sensitivity of the flow cytometric analysis, the close proximity requirement is removed. This is done by letting the RCA event take place only using one probe, a technique called Immuno RCA (ImRCA).

As can be seen from figure 17 the cell sample that has not been subjected to primary antibodies displays a rather high number of signals when analysed using a fluorescence microscope. Theoretically one would expect no signals since the probe used for the RCA event has no binding target. This result suggests that ImRCA give rise to a high number of false positives. From the figure, one can also note that cell sample subjected to primary antibody gives a high average number of signals per cell.

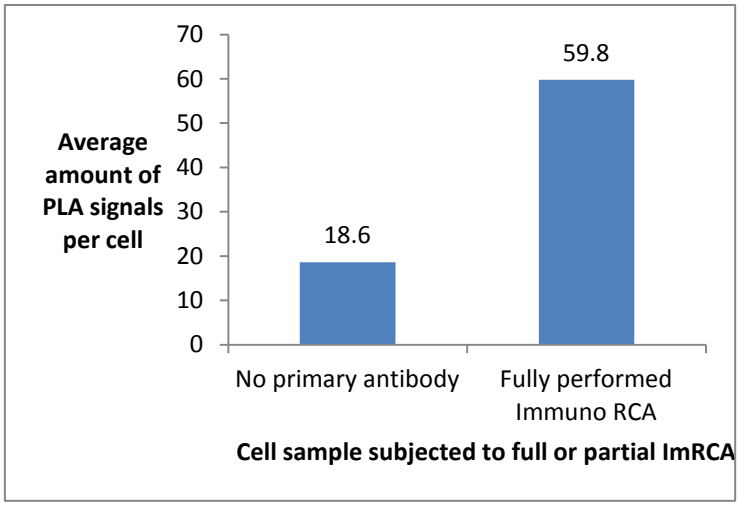


Figure 17. *Overview of average number of ImRCA-signals per cell, when cell samples have been subjected to full or partial Immuno RCA. Fully performed ImRCA give rise to a large amount of signals per cell.*

When analysing the cell samples using flow cytometry, the impact that false positives has on the analysis is obvious. As can be seen from figure 18, a large amount of the cells not subjected to any primary antibodies are registered as rhodamine positive cells. Since we do know that no ImRCA-signals should be present in those cells, it can be concluded that false positives do lower the sensitivity of the flow cytometric analysis. Thus, the usage of *in situ* PLA as protein detection method strengthens the precision and accuracy of the flow cytometric analysis.

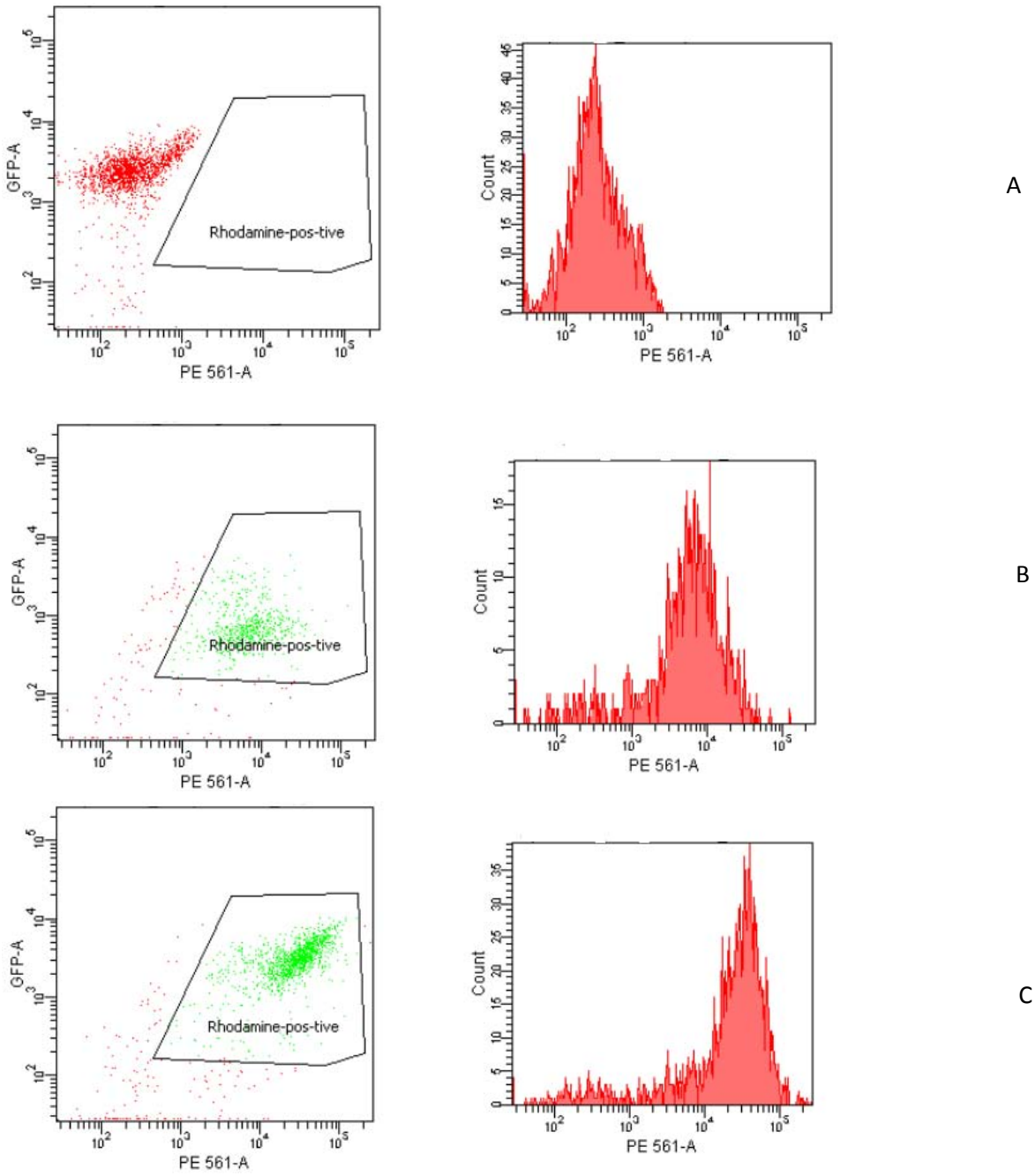


Figure 18. *Overview of numbers of events registered as cells containing PLA-signals, diagrams to the left. Here the fluorescent intensity of used fluorophore can be seen on the x-axis, and the GFP fluorescens can be seen on the y-axis. The total mean fluorescence of specified fluorophore for the cell population can be seen in diagras to the right. Here the fluorescent intensity of used fluorophore can be seen on the x-axis, and number of cells on the y-axis. A large number of false positives can be noted when partial ImRCA is performed.*

A) Cells not subjected to any ImRCA-events
B) Cells subjected to ImRCA, but no primary antibody added
C) Cells subjected to complete ImRCA

4 Discussion

In situ PLA is a method that usually is performed on fixed cells attached to glass slides, or on FFPE tissues. When moving from cells attached to surfaces to cells free in solution, it has been claimed that the main concerns relates to cell retrieval (21, 25), e.g. how to maintain as much of the cells in the sample as possible throughout the whole experiment. In an attempt to solve this problem two methods, based on centrifugation and filtration, were investigated. When wanting to discard reagents, add new solutions or wash samples, all events that are exercised when performing *in situ* PLA, it must be possible to separate cells from the surrounding solution. By decreasing the risk for potential cell loss, the risk for incorrect diagnosis will also decrease. Hence, the separation technique used must be as effective as possible.

By centrifuging the sample a cell pellet may be formed, allowing one to discard the supernatant and by that the separation between cells and solutions. Since the cells are to be analyzed using a flow cytometer, the cells must be maintained intact. This restricts the centrifugation technique, since speed and time of centrifugation may damage the cells. Also, the rigidity of the formed pellet may affect the efficiency of the method which has an impact on the cell retrieval. All of those constraints contribute to problems concerning automatisation, hindering the possibilities for high throughput diagnosis. Since the formation of pellet, when centrifuging cell samples, partially depends on the type of cells that are analysed, it is hard to describe a standard of how much supernatant that can be removed without disturbing the pellet. By restrictions in the formation of standard performances, limitations in high throughput analysis are obvious. However, by choosing centrifugation as a separation method gives us the possibility to screen larger amount of cells at one time, meaning that centrifugation might be the choice of separation method when wanting to analyse large amount of cell sample.

When performing filtration of the sample in order to separate cells from the solution, the pore size determines how well the cells are separated from any reagents that may be present in the solution. By using filters with a pore size small enough not to let any cells through, but large enough to easily allow reagents and liquids to pass, cells can be maintained on top of the filter when draining the solution. Here the cell retrieval is determined by the cells ability to adhere to the surface of the filter, as well as the rigidity of the cells. Using filtration as a separation method does however create a bottleneck concerning amount of cells in sample. Since the separation method demands unclogged filters in order to efficiently separate cells from surrounding solution, there is a restriction in the amount of cells that can be investigated per filter area. Hence, this separation method opens up possibilities for high throughput analysis, but only for smaller samples.

Thus, by the ability to use two different separation methods customized for samples containing either small or large amounts of cells, allows usage of the *in situ* PLA method for a wide variety of samples. Also, the risks of losing cells during washing steps have been proven not to be imminent.

When analysing the samples with flow cytometry, the intensity as well as the amount of resulting PLA-signals play a vital role. In order to maximize the amount of PLA-signals, the quantity of reagents must be proportional to amount of cells in the sample. That is, the amount of reagents added to the sample must be adequate with regards to amount of protein target present in every cell. In order to investigate what amount of reagents that contributes to a resulting maximum of PLA-signals, 240 000 cells were subjected to different amounts of reagents. The concentrations of reagents in mixtures,

provided by Olink Bioscience, used for *in situ* PLA were maintained. The amount of reagents added to each cell sample was thereby only controlled by varying the reaction volumes. Here one could clearly see that larger reaction volumes did not contribute to more signals. Also, one could see that smaller reaction volumes gave rise to fewer signals. Hence, the amount of reagents strictly controls the amount of resulting signals when performing *in situ* PLA on cells in solution. However, the limiting reagent has not been yet been identified. Research has previously shown that *in situ* PLA can be performed on cells in solution when using much lower amounts of Duolink reagents than what is used in the optimized reaction volume (16). With that information taken in to account, one can suspect that the used primary antibody may be working as the bottleneck in the overall reaction. By increasing the total amount of primary antibody, done by increasing the overall reaction volume, one also increases the resulting PLA-signals. By choosing a new antibody that has a higher affinity towards the target of interest, one might be able to decrease the reaction volume. Since amount of PLA-signals directly contribute to the total mean fluorescence intensity of the cell population, suitable reaction volumes will invigorate the sensitivity of the analysis.

When using flow cytometry as a readout method, the total fluorescent intensity in every cell is measured. Compared to the normal analysing method, usage of a fluorescence microscope where number of individual fluorescent signals are counted, the resulting data is of a different kind. In order to observe an increase / decrease of the total fluorescence, the amount of signals as well as the intensity of the existing signals are essential. Those are the factors that determine to what extent we can distinguish between true and false positives, as well as between cells that are containing PLA-signals and those that do not. This means that by identifying as many target proteins as possible, and / or to produce high intensity PLA-signals, cells containing more or less of a protein of interest may be distinguished from other cells.

In order to produce as many signals as possible, one would have to be sure that all reagents reach their intended target. The first barrier that the reagents are encountering is the cell membrane. In order to allow for large reagents, such as antibodies and enzymes, to enter the cell, the outer cell membrane has to be punctured. When permeabilizing cells, the amount of detergent, in this study Triton has been used, is of importance. Too much detergent will efficiently penetrate the membrane and antibodies will easily reach their intended targets, but there is a risk that the cell will rupture. Hence, too much detergent may decrease the cell retrieval. However, too low concentrations of detergent may instead not permeabilize the membrane efficiently enough, making it difficult for antibodies to enter cells. By combining the optimized reaction volumes with respect to amount of cells in the starting sample with an efficient permeabilization, one has maximized the amount of PLA-signals that can be produced. However, only one cell type has been investigated and one must take in to consideration that other cell types may demand other types of treatment in order to obtain larger amounts of PLA-signals and at the same time maintain good cell retrieval.

It has previously been shown that cells containing approximately 30 signals per cell or more can be separated from cells containing around 5 signals, by flow cytometric analysis (21). Cells containing the larger part of the signals here display about 10 times as high intensity compared to cells containing the lower amount of signals. By increasing the intensity of the maximized number of PLA-signals, an easier detection of cells containing PLA-signals can be achieved. In order to increase the intensity, one would wish to raise the amount of fluorophores attached to the RCA-product. Throughout this degree project I have shown that this can be accomplished by an increase in size of

the RCA-product, creating more areas to which detection oligonucleotides can hybridize. In this report it has been demonstrated that by increasing the intensity of the total fluorescence, achieved by increasing the amount of fluorophores on every RCA product, an easier distinction between cells containing PLA-signals and cells that do not, is reached. It has also been shown that this enlargement of the RCA-product increases the sensitivity of the method, allowing for detection of non-abundant proteins and separation between cells with and without that particular type of protein. Here the intensity is about five times higher for cells containing in average 1.5 signals compared to cells containing none. Despite the unexpected average of signals per cell received using a dilution series of the primary antibody, the conclusion remains unchanged. Even though no stepwise decrease in signal could be seen, it is still clear that cells containing in average 1.5 signals can be separated from cells containing none.

The increase in total fluorescence from every cell is obtained by increasing the size of the RCA product. However, the fluorescence intensity of the cells also depends upon the fluorophores used. As has been shown, the intensity of light emitted from used fluorophore restricts the sensitivity of the flow cytometric analysis. A highly sensitive analysis demands a larger distinction between cells containing PLA-signals and those that do not, i.e. the analysis demands PLA-signals emitting high intensity light. Hence, by choosing a fluorophore that with a high quantum yeild, the overall sensitivity of the diagnostic method is increased.

The need for probes in close proximity in order to form an RCA product, has earlier been proven to lower the amount of false positives when performing *in situ* PLA (18). During this degree project it has been shown that this particular event is crucial for the sensitivity of the flow cytometric analysis. Performing ImRCA on cells in solution, instead of *in situ* PLA, creates a large number of signals in cells that should contain none, the negative control. This is clearly visible when using flow cytometry as an analysing method. A large portion of the negative control is registered as true positives although all of them clearly are false positives. It is achievable to distinguish between cells containing only false positives and those that are containing true positives, if the amount of target allows for a great amount of signals per cell. This is due to the increase in mean total fluorescence in the cell population containing true positives. However, the false positives harshly reduce the sensitivity of the analysis. Since all signals, true or false positives, are registered when performing a flow cytometric analysis, low abundant proteins or minor increases / decreases in protein levels are not able to be detected. This since the results is overshadowed by the false positive signals.

In this study, it has been shown that performed *in situ* PLA on cells in solution do generate approximately as much PLA-signals inside cells that the conventional method generates. Hence, the fixation of cells onto glass slides before performed *in situ* PLA does not generate more signals. However, the study showed that the amount of signals that could be detected on the glass slide itself, now called the background, increased 150 fold when performing the *in situ* PLA on fixated cells on glass slides. Therefore it might be more favourable to always perform the protein detection method on cells in solution, and thereafter fixate them onto a glass slide.

By showing that it is possible to detect proteins using single recognition when performing *in situ* PLA on cells in solution, one can assume that this method also will work when using dual recognition. Hence, this method should be able to detect both proteins and protein interactions.

5 Materials and methods

Performing *in situ* PLA on cells in solution with flow cytometric readout demands a new workflow. This since *in situ* PLA only is performed on cells fixated on glass slides or on FFPE tissue today. In order to maintain as many cells as possible throughout the whole experiment, two techniques, filtration and centrifugation, were evaluated. Also protocol optimisations concerning usability and efficiency were investigated. The following section is divided into five main parts describing fixation of cells used, the workflow for *in situ* PLA performed on cells in solution, the workflow for in situ PLA performed on cytospin samples, the workflow for ImRCA on cells in solution and analysis.

5.1 Fixation of U937 cells

Throughout the whole degree project the cell line U937, human histiocytic lymphoma, was used. Upon arrival of the ordered cell line (3H Biomedical, Sweden), fixation was performed. U937 suspended in growth media was centrifuged (200 x g) for five minutes without retardation. Supernatant was discarded and the cell pellet was washed once in cold phosphate buffered saline (PBS). The cell suspension was centrifuged again (200 x g) without retardation for five minutes. Fixation of the cells was performed overnight at +4°C by adding 3% paraformaldehyde (PFA) in PBS. Fixated cells were stored at +4°C up to one week.

5.2 Performing *in situ* PLA on cells in solution

In order to counteract unspecific binding of antibodies to PFA molecules, blockage of PFA was performed by adding 1 M glycine to the solution containing fixated cells. U937 cells were then separated from surrounding solution using one of the possible separation methods, filtration or centrifugation. The same separation method was then used throughout the whole experiment before every wash and addition of new reagents. After the separation, cells were washed once in PBS and then permeabilized for 30 minutes at + 4°C using 0.2% Triton X-100 in PBS. After permeabilization, cells were washed once with PBS and Duolink blocking (Olink Bioscience, Sweden) was added in a concentration of 7 drops per 240 000 cells. The blocking was performed at +37°C for 30 minutes and was executed in order to block unspecific binding between antibodies and non-target molecules.

Primary antibodies, ab11005 FITC conjugated mouse monoclonal antibodies directed towards actin, (Abcam, England), diluted to a final concentration of 12 µg/ml in Duolink antibody diluent (Olink Bioscience,) was added to the cells and the mixture was incubated at +37°C for 45 minutes or at +4°C overnight. The primary antibody ab3280 mouse monoclonal antibody, directed towards actin, (Abcam) was used in experiment where different fluorophores were tested. Here a dilution series of the primary antibody were performed and the final concentrations of 1000, 125, 62.5, 31.3, 15.6 and 7.8 ng/ml of the primary antibody, diluted in Duolink antibody diluents (Olink Bioscience) were used. Since the primary antibody is directed towards actin, a target that is abundant in cells, the primary antibody was significantly diluted in order generate PLA-signals that are separated from each other. This to make sure that each signal can be counted when analyzing resulting samples using a fluorescence microscope. Cells were then washed once in Duolink washing buffer A (Olink Bioscience) for 5 minutes. 250 µl per 240 000 cells were used. Proximity probes, anti-mouse plus and minus (Olink bioscience), directed towards the primary antibody, and diluted to a final concentration of 2,5 µg/ml in Duolink antibody diluent (Olink Bioscience), were added to the cells. The mixture was incubated for 1 hour at +37°C. After incubation the cells were washed once in Duolink washing buffer A (Olink Bioscience) for 5 minutes with a volume of 250 µl per 240 000 cells.

When unbound proximity probes had been washed away, a mixture of ligation solution, T4 DNA ligase (all reagents from Olink Bioscience) and water was added. The solution was incubated for 30 minutes at +37°C and thereafter washed once for 2.5 minutes, 250 µl per 240 000 cells, with Duolink washing buffer A (Olink Bioscience). When the formation of the DNA circle was completed, amplification solution, phi29 polymerase (all reagents from Olink Bioscience) and MQ water was added and the solution was incubated for 100 minutes or up to 4 hours at +37°C, in order to form RCA products. After amplification the cells were washed once, 250 µl per 240 000 cells, in Duolink 1 x washing buffer B (Olink bioscience) for 5 minutes and then washed again for 5 minutes in 0.01 x washing buffer B using 250 µl per 240 000 cells. For analysis using a fluorescent microscope, 10µl of cell suspension was dried onto a glass slide in room temperature over night or at +37°C for 1 to 2 hours. Remaining cells were separated from surrounding solution and suspended in 550 µl PBS.

5.2.1 Filtration or centrifugation as a separation method

For smaller samples, filtration can be used as a separation method. To one Nanosep® tube, with pore size of 0.2 µm (Pall life sciences, U.S.A.), up to 320.000 cells were added. Centrifugation performed in fixed angle centrifuge at 500 x g for 1 minute is sufficient to filter away up to 500 µl solution from the cells.

For samples containing large amount of cells, centrifugation is preferred as a separation method. A minimum amount of 370 000 cells was added to one 1.5 ml eppendorf tube and PBS was added, so that the total volume of the mix reached 1 ml. By centrifuging the tube in a fixed angle centrifuge at 300 x g for 10 minutes a pellet was formed, allowing for removal of the supernatant. Longer centrifugation time, such as 10 minutes, gives more visible pellet formation than shorter centrifugation time, when centrifuging with a speed of 500 x g.

5.3 *In situ* PLA performed on cytospin sample

Cytospin sample (3H Biomedical) containing two areas of U937 cells with around 75 000 cells each were permeabilized using 150 µl 0.2% Triton X-100 in PBS for 3 minutes at room temperature. The glass slide was then washed in PBS for 3 minutes under gentile agitation. In order to block unspecific binding between and non-target molecules, 4 drops of Duolink blocking (Olink Bioscience) was added to each area. The sample was incubated for 30 minutes at +37°C.

After incubation, the excess fluid was gently removed and primary antibody, ab11005 FITC conjugated mouse monoclonal antibodies directed towards actin (Abcam), was added at a concentration of 12 µg/ml after being diluted in Duolink antibody diluent (Olink Bioscience). The glass slide was incubated for 45 minutes at +37°C. After incubation, the slide was washed twice in Duolink washing buffer A (Olink Bioscience) at room temperature for 2 x 5 minutes.

After the washes, the sample was subjected to 150 µl of proximity probe solution. Proximity probes, anti-mouse plus and minus (Olink bioscience) directed towards the primary antibody, were diluted to a final concentration of 2,5 µg/ml in Duolink antibody diluent (Olink Bioscience). The cytospin was incubated for 1 hour at +37°C. After incubation, the slide was washed twice in Duolink washing buffer A (Olink Bioscience) at room temperature for 2 x 5 minutes.

In order to ligate the connector oligonucleotide, the sample was subjected to ligation solution, T4 DNA ligase (all reagents from Olink Bioscience) and water and thereafter incubated for 30 minutes at

+37°C. Reagents were washed away after incubation by two washes in Duolink washing buffer A (Olink Bioscience) at room temperature for 2 x 5 minutes.

The RCA product was formed by adding amplification solution, phi29 polymerase (all reagents from Olink Bioscience) and MQ water to the sample. The glass slide was then incubated for 100 minutes in +37°C. All unwanted reagents were then washed away by two washes in Duolink 1 x washing buffer B (Olink bioscience) for 2 x 10 minutes and then one wash in 0.01 x washing buffer B (Olink Bioscience) for 1 minute. The sample was then dried for 2.5 days in room temperature, in the dark.

5.4 Workflow for performance of Immuno RCA on cells in solution

The workflow for ImRCA on cells in solution is similar to the one for *in situ* PLA on cells in solution. Deviations such as preligated oligonucleotide circle to used plus proximity probe, and thereby the reduction of the ligation step, is performed.

A pre-ligation of connector oligonucleotide, forming the oligonucleotide circle, to one plus probe was performed by mixing 0.5 µl of the connector oligonucleotide with a concentration of 10 µM with 50 µl of the proximity probe anti-mouse plus 125 µg/ml, 20 µl Ligation stock, 1.25 µl T4 DNA ligase and 28.5 µl MQ water (all reagents provided by Olink Bioscience). The proximity probes used were concentrated 10 times using Nanosep ® 30K OMEGA (Pall life sciences) before used in the pre-ligation. The pre-ligation mixture were incubated at +37°C for 30 minutes and then stored in +4°C for a minimum of 4 days before use.

The pre-ligated probe was diluted five times just before usage by mixing 20 µl of the pre-ligated probe with 80µl MQ water.

5.5 Analysis of assay performance

When cells had undergone *in situ* PLA reaction, they were analysed using both a fluorescent microscope as well as a flow cytometer. Pictures of cells were taken using a Zeiss Axio imaging M1 fluorescence microscope. Three fluorescence filters were used to detect DAPI, Rhodamine and FITC. Images were analyzed, counting number of cells and PLA-signals, using the software program ImageTool. Before analysis of glass slides using the Fluorescence microscope, the glass slides were mounted using Duolink mounting medium with DAPI (Olink Bioscience).

For the analysis of the total PLA-signal fluorescence in individual cells, the flow cytometer BD LSR II was used. Resulting measurements were analyzed in the software program BD FACSDiva.

When estimating the efficiency of the workflow concerning the cell retrieval, 20 pictures of 10 µl of cell suspension was taken. References were produced by separating fixed cells from PFA by chosen separation method and then dilute the cells in 1 x Washing buffer B (Olink Bioscience). Cells were then counted in every picture and a mean value of cells per picture was calculated.

6 Conclusion

In this report I have demonstrated the ability to perform *in situ* PLA on cells in solution and use flow cytometry as an analysing method. By using centrifugation or filtration as separation methods, I have shown that the cell loss can be minimized allowing for a good cell retrieval after performed *in situ* PLA protocol. Also, the average amount of signals per cell has been proven to be as good when performing *in situ* PLA on cells in solution as when being performed on cells fixated onto glass slides. The false positive signals on glass slides has drastically been reduced when performing the protein detection method on cells in solution, suggesting that *in situ* PLA performed on cells in solution has a higher dynamic range.

Protocol optimisations have contributed to an increase in the sensitivity of the analysis. By optimizing amount of reagent added to every cell, the availability of actin for the primary antibody and by increasing the intensity of the signals, the methods permit for a good resolution. This enables a distinction between cells containing as few as 1.5 signals compared to cells containing none, in one complex sample. Due to this good resolution, the flow cytometric analysis allows for detection of every PLA-signal present in cells. Hence, flow cytometric analysis delivers results that are as good as results delivered by fluorescence microscope.

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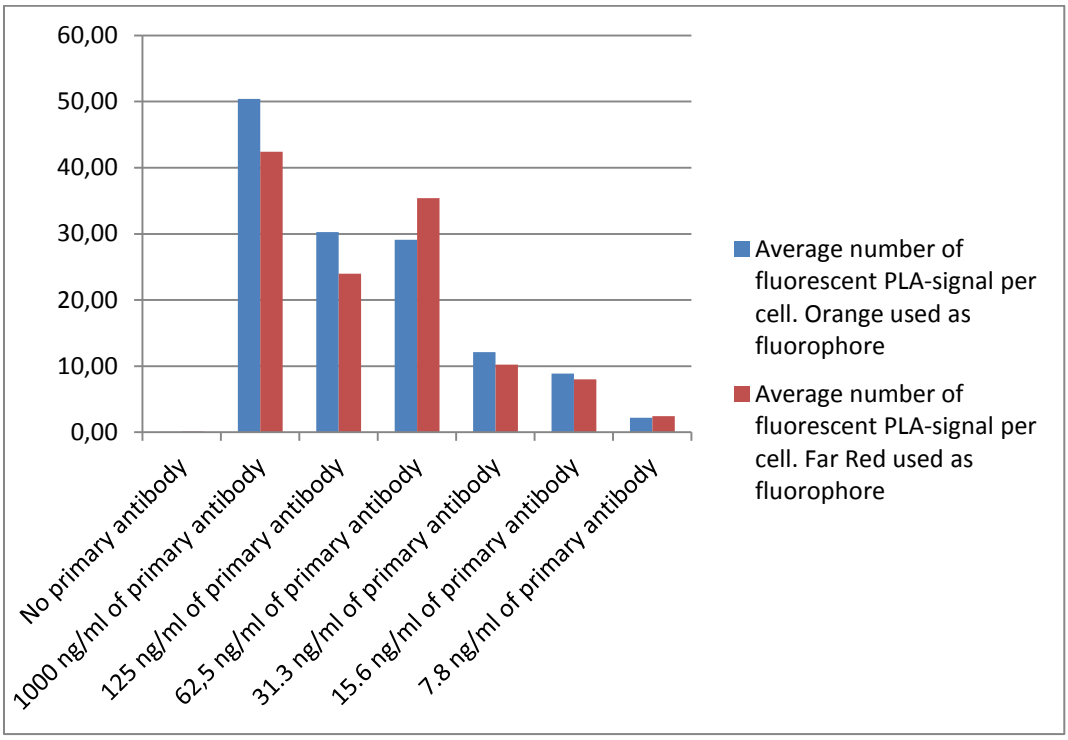
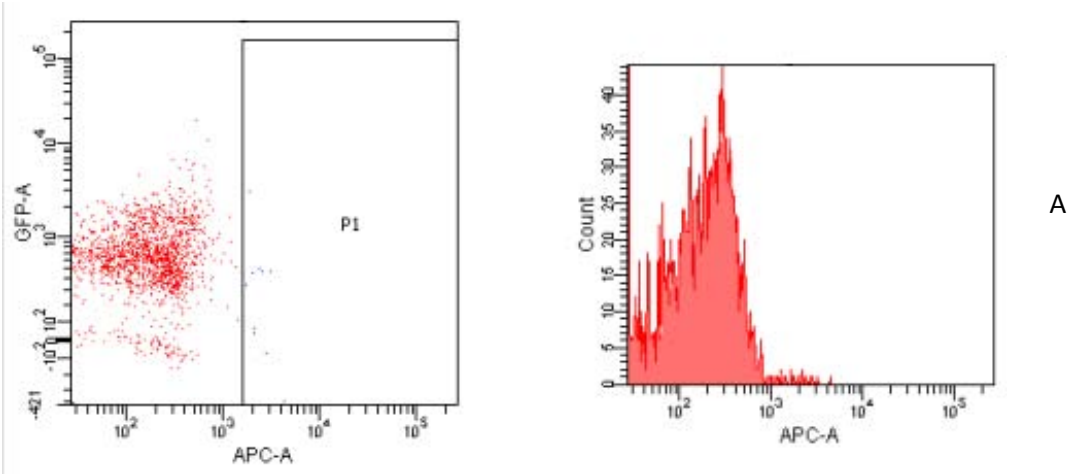
9 Supplementary data

Supplement 1S



Supplement 1S. *Figure presenting average number of PLA-signals per cell when cell sample have been analysed using a fluorescent microscope. Regardless of fluorophore used, approximately the same amount of PLA-signals per cell could be noted.*

Supplement 2S



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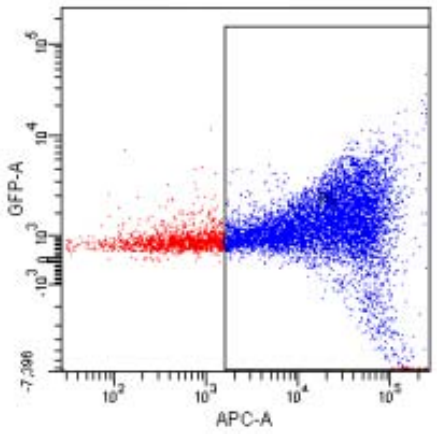
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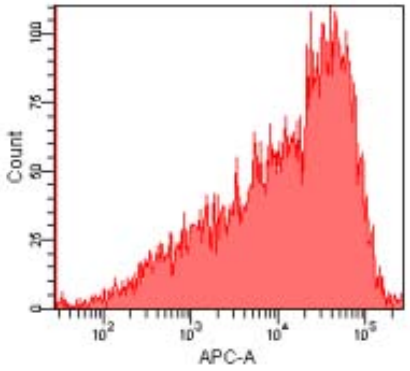
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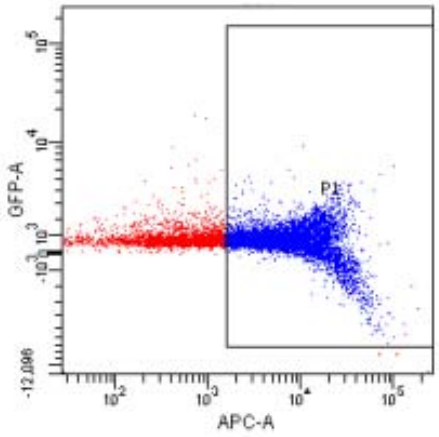
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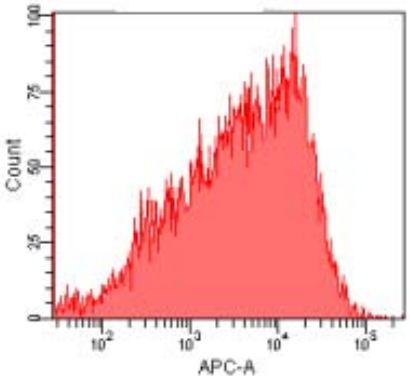
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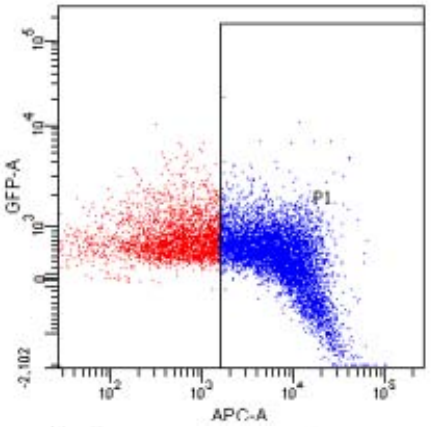
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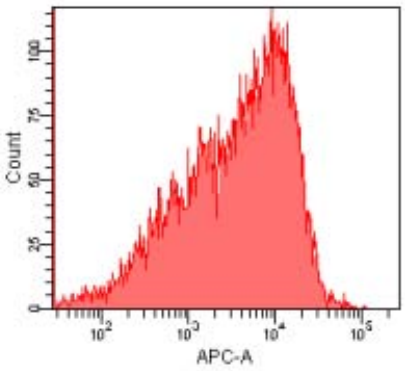
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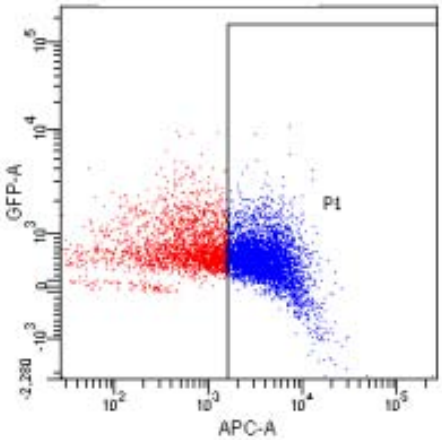
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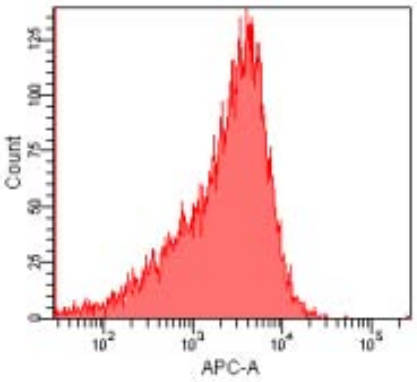
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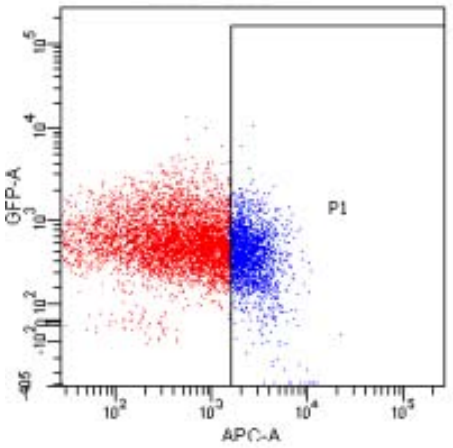
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P1
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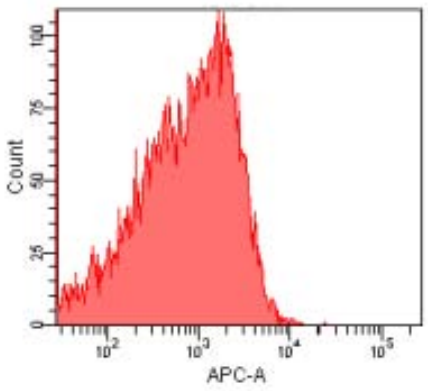
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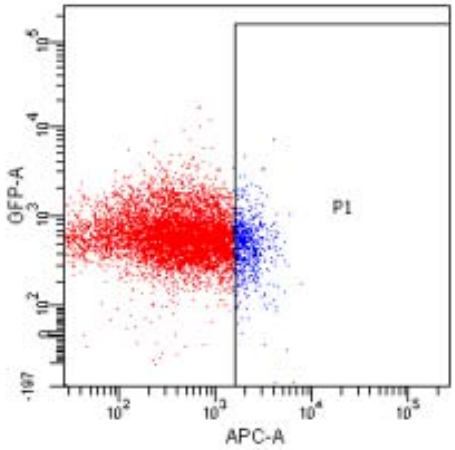
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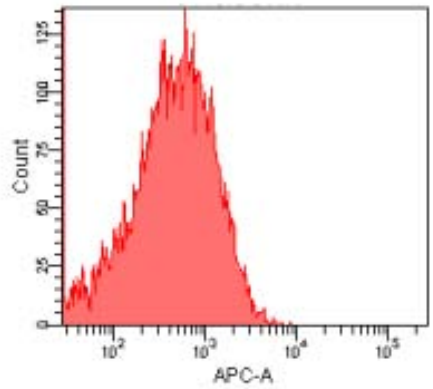
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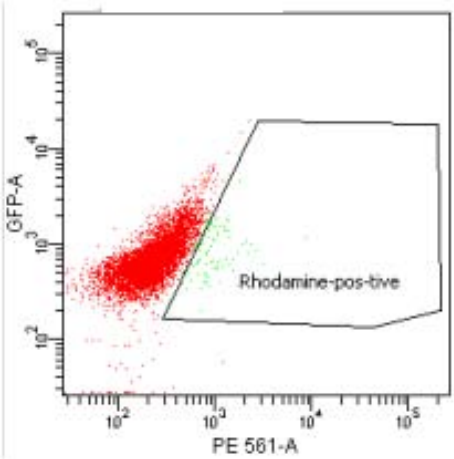
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Rhodamine-pos-tive
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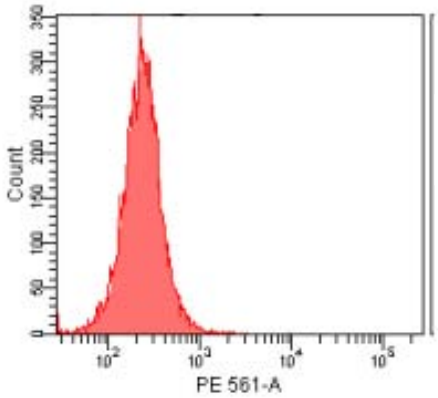
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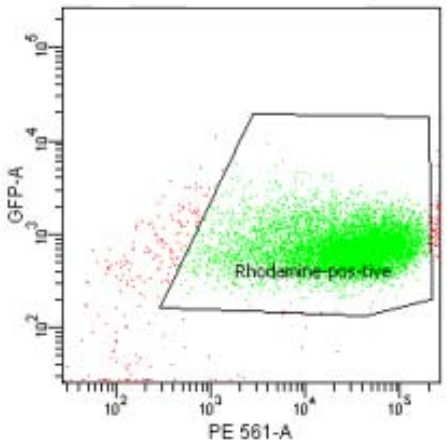
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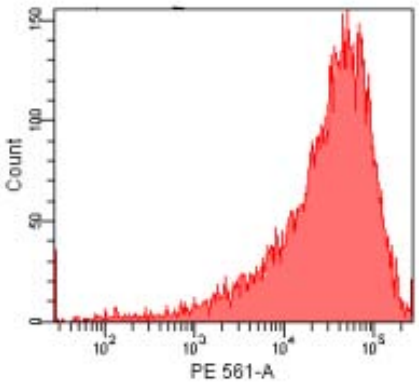
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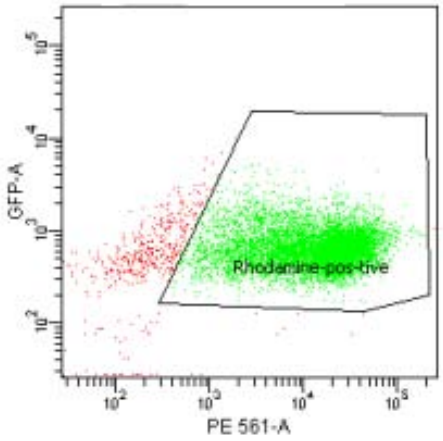
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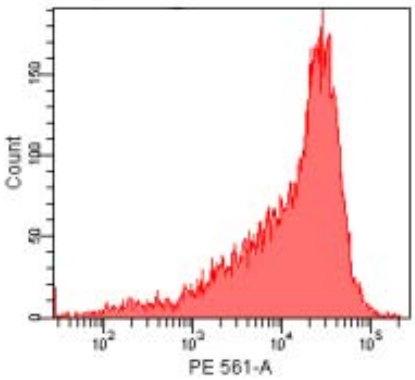
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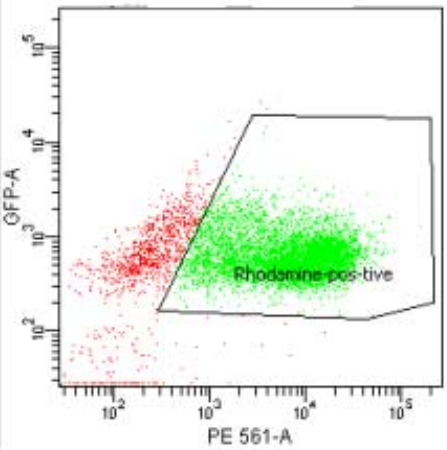
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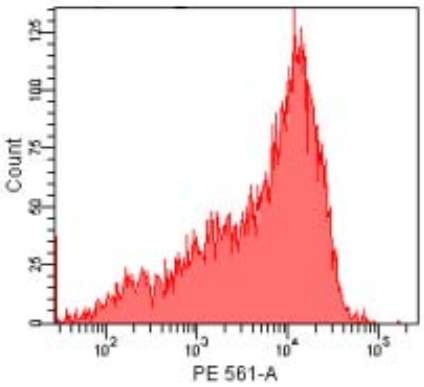
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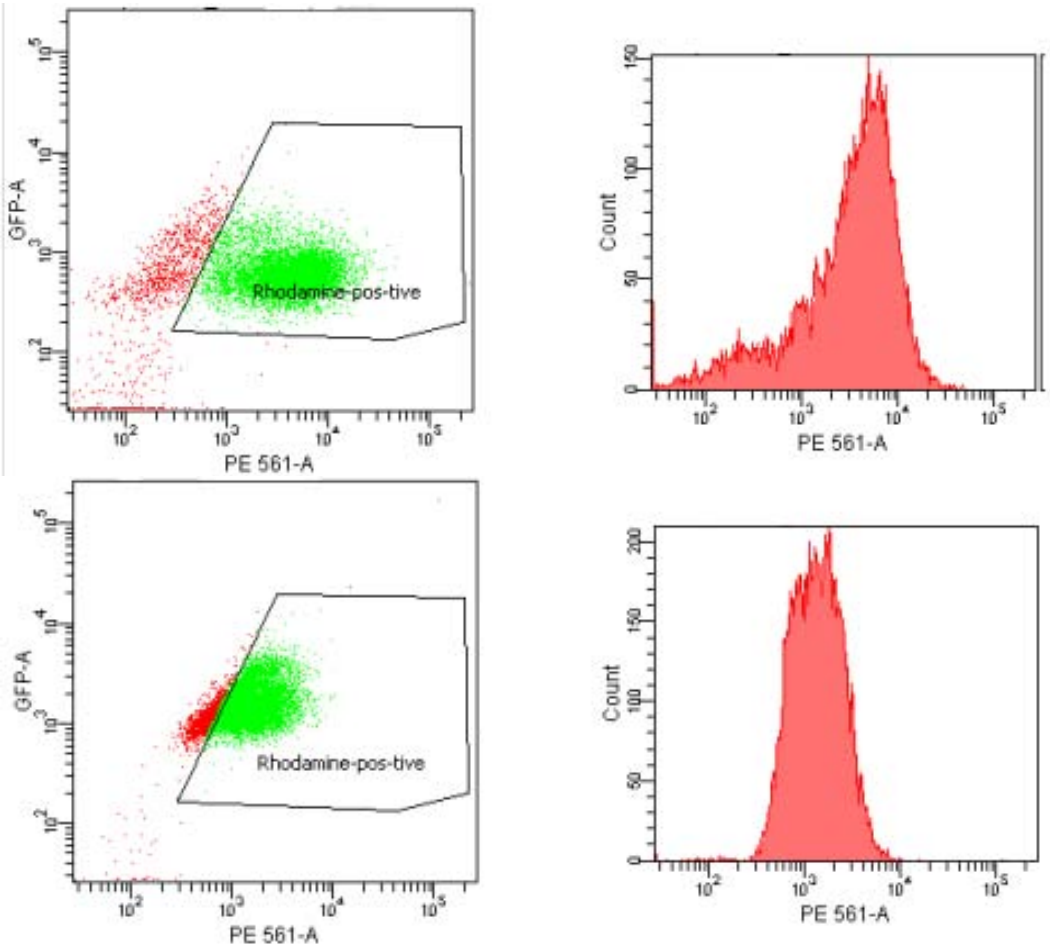
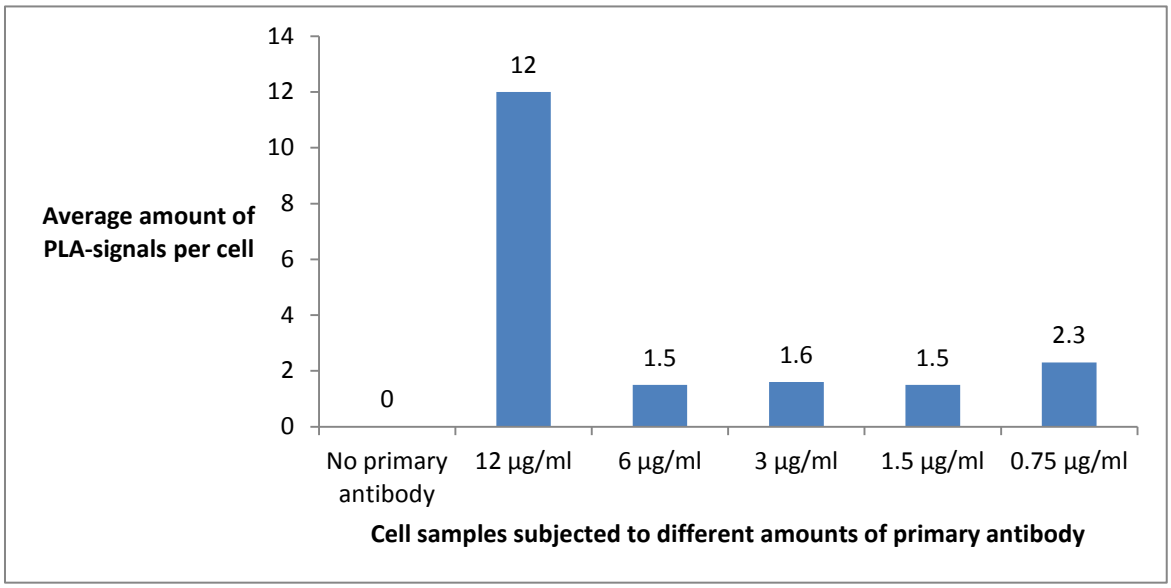


Supplement 2S. *Overview of numbers of events registered as cells containing PLA-signals, diagrams to the left, and mean total fluorescent intensity of the cell population, diagrams to the right. In diagrams to the left, the fluorescent intensity of used fluorophore can be seen on the x-axis, and the GFP fluorescens can be seen on the y-axis. In diagrams to the right, the fluorescent intensity of used fluorophore can be seen on the x-axis, and number of cells on the y-axis. All cell samples have been subjected to full in situ PLA protocol. Comparison of used fluorophores, Far Red and Orange, is performed. The fluorophore Far Red gave rise to a large number of false negatives. This could be concluded since samples subjected to the same amount of primary antibody had approximately the same amount of PLA-signals regardless of fluorophore used (Data in supplement 2S).*

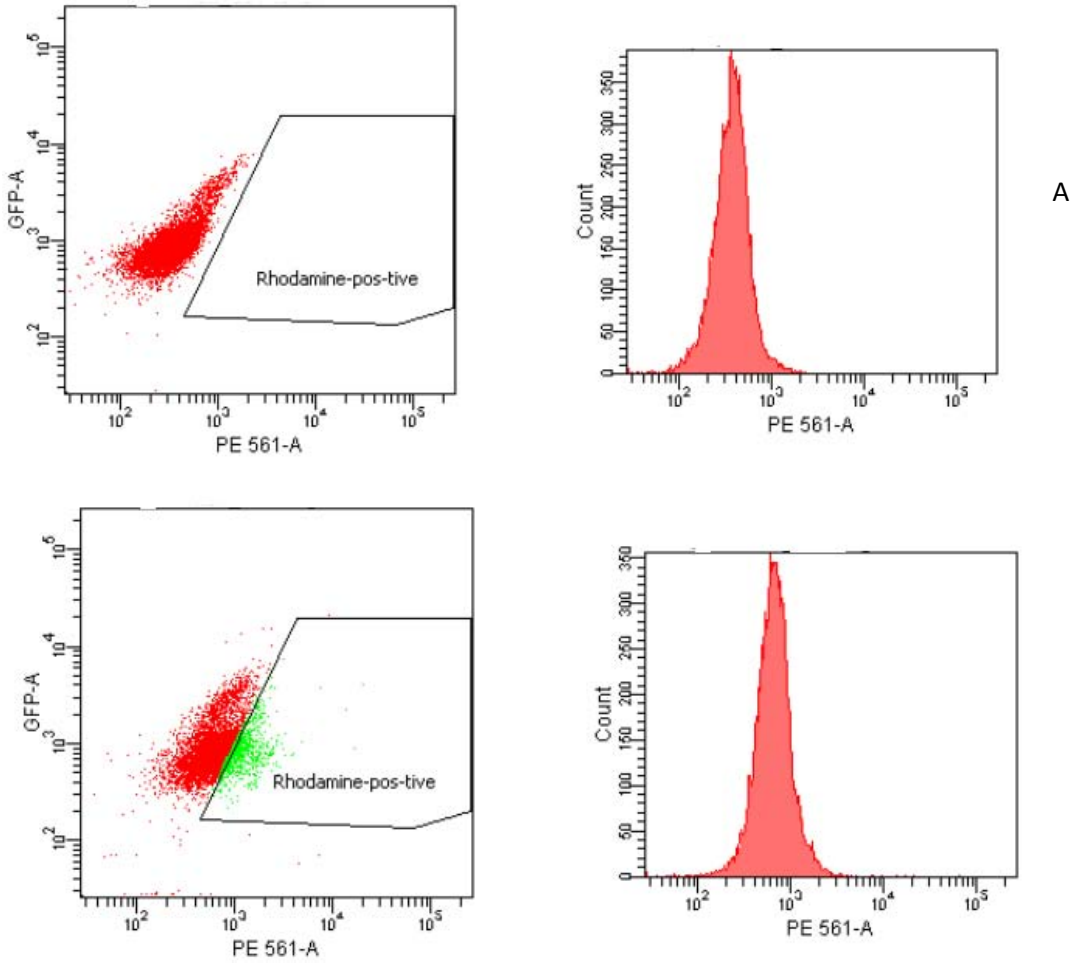
- *A) Cell sample not subjected to primary antibodies. Used fluorophore is Far Red*
- *B) Call sample subjected to 1000 ng/ml of primary antibody. Used fluorophore is Far Red*
- *C) Call sample subjected to 125 ng/ml of primary antibody. Used fluorophore is Far Red*
- *D) Call sample subjected to 62.5 ng/ml of primary antibody. Used fluorophore is Far Red*
- *E) Call sample subjected to 31.3 ng/ml of primary antibody. Used fluorophore is Far Red*
- *F) Call sample subjected to 15.6 ng/ml of primary antibody. Used fluorophore is Far Red*
- *G) Call sample subjected to 7.8 ng/ml of primary antibody. Used fluorophore is Far Red*
- *H) Call sample subjected to 1000 ng/ml of primary antibody. Used fluorophore is Orange*
- *I) Call sample subjected to 125 ng/ml of primary antibody. Used fluorophore is Orange*
- *J) Call sample subjected to 62.5 ng/ml of primary antibody. Used fluorophore is Orange*
- *K) Call sample subjected to 31.3 ng/ml of primary antibody. Used fluorophore is Orange*
- *L) Call sample subjected to 15.6 ng/ml of primary antibody. Used fluorophore is Orange*
- *M) Call sample subjected to 7.8 ng/ml of primary antibody. Used fluorophore is Orange*

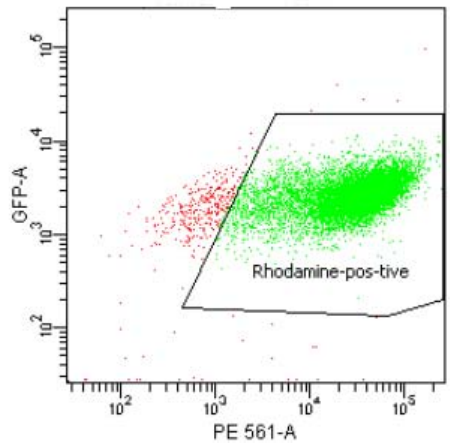
Supplement 3S

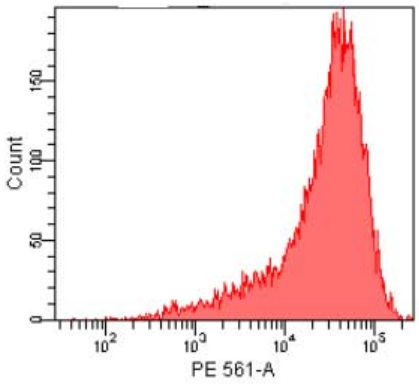


Supplement 3S. *Figure describing average amount of signals per cell when cell samples have been subjected to different amounts of primary antibody. As can be noted, the average amount of PLA-signals per cell does not display a linear decrease in signals, as was expected.*

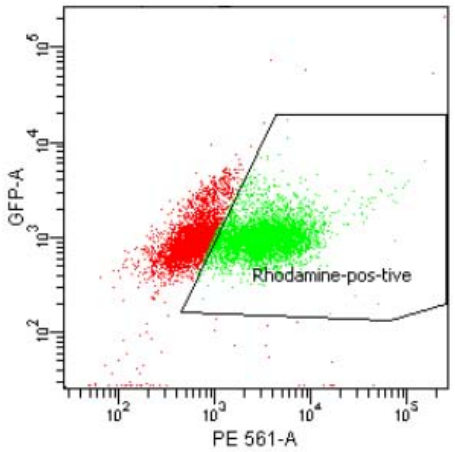
Supplement 4S




GFP-A
Rhodamine-pos-tive
PE 561-A


Count
PE 561-A

C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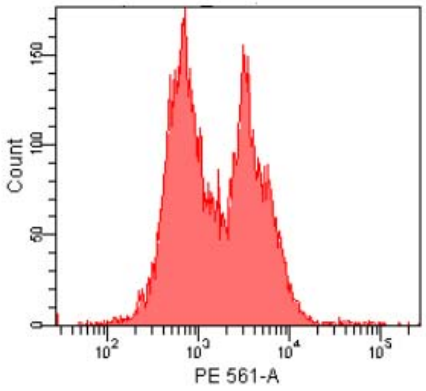
Rhodamine-pos-tive
PE 561-A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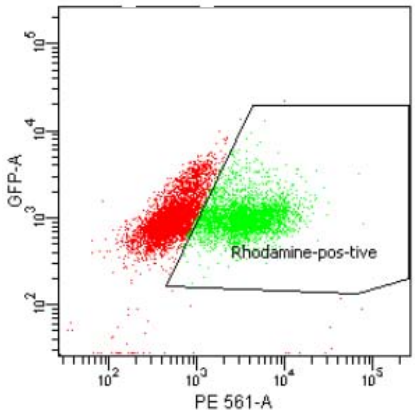
PE 561-A

D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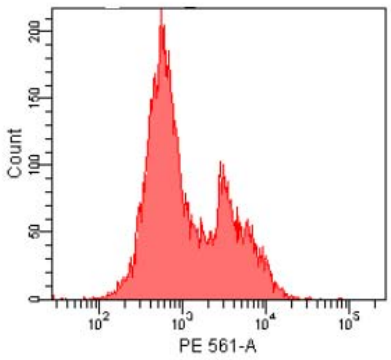
Rhodamine-pos-tive
PE 561-A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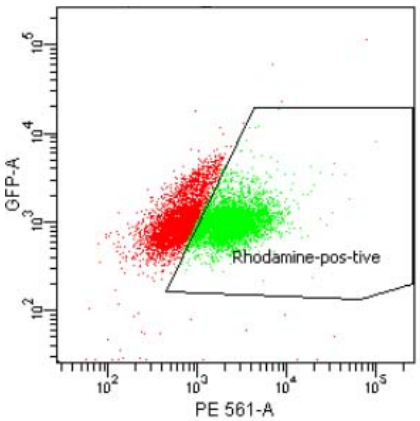
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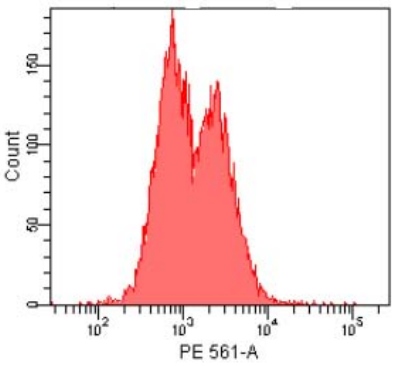
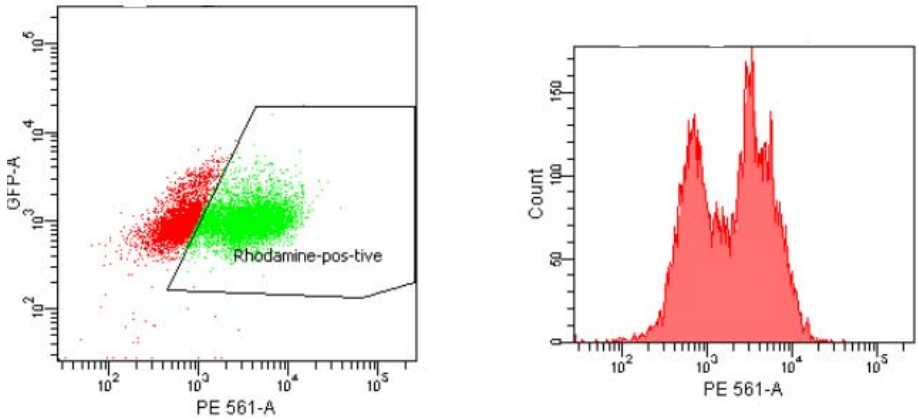
Rhodamine-pos-tive
PE 561-A


Count
PE 561-A

F



G

Supplement 4S. *Overview of numbers of events registered as cells containing PLA-signals, diagrams to the left, and mean total fluorescent intensity of the cell population, diagrams to the right. In diagrams to the left, the fluorescent intensity of used fluorophore can be seen on the x-axis, and the GFP fluorescens can be seen on the y-axis. In diagrams to the right, the fluorescent intensity of used fluorophore can be seen on the x-axis, and number of cells on the y-axis. Investigation of the sensitivity of the flow cytometric analysis is performed. Mean PLA-signals per cell for the different samples can be seen in complementary data 3S. In 4S, a large variation of amount of cells not containing any PLA-signals can be seen. This is not registered in 3S. However, one can note a destinction between cells containing on average less than 2 signals per cell from cells containing no signals. Thus, the sensitivity of the analyzing method is high.*

- *A) Cells not subjected to any PLA-events at all*
- *B) Cells not subjected to primary antibody*
- *C) Cells subjected to full PLA reaction. 12 µg/ml of primary antibody was used*
- *D) Cells subjected to full PLA reaction. 6 µg/ml of primary antibody was used*
- *E) Cells subjected to full PLA reaction. 3 µg/ml of primary antibody was used*
- *F) Cells subjected to full PLA reaction. 1.5 µg/ml of primary antibody was used*
- *G) Cells subjected to full PLA reaction. 0.75 µg/ml of primary antibody was used*