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Advanced molecular tools for diagnostic analyses of RNA and antibodies in situ and in solution

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Abstract

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Advanced molecular diagnostics uses in vitro biological assays to detect nucleic acids or proteins even in low concentrations across samples, allowing for the identification of biomarkers, monitoring the course of the disease over time, and selection of appropriate therapy. In this thesis, I focus on development and early applications of several molecular tools of expected value in research, and eventually also clinically.

In papers I and II, proximity extension assay (PEA) was for the first time modified to measure specific antibody responses, rather than protein levels as in the standard PEA. We call the method AbPEA and the technique was used to sensitively measure antibody responses to the spike protein or the nucleocapsid of SARS-CoV-2. We demonstrated that AbPEA has high specificity, sensitivity, and broad dynamic range, along with multiplexing potential, offering performance similar to that of other methods for antibody measurements. We demonstrated utilization of blood and saliva samples in paper I and paper II, respectively, which further establish that our approach has great potential for large-scale screening and biobanking.

In paper III, we aimed to investigate how the protein composition of extracellular vesicles (EVs) differed among blood samples collected from healthy individual or ones with either mild or severe COVID-19. Proximity barcoding assay was applied to obtain a comprehensive overview of the protein composition of large numbers of individual EVs, demonstrating interesting differences.

In paper IV, we enhanced padlock-RCA-based RNA genotyping in situ by using another newly developed technology for highly selective detection of DNA or RNA sequence variants, referred to as super RCA (sRCA). Our analysis showed that this approach can improve the selectivity for sequence variants during in situ detection of mutant or wild-type transcripts, and the signals representing superRCA reaction products are prominent and easily distinguished from any background.

Keywords: Homogenous serological assay, PCR-based antibody detection, Proximity extension assay, SARS-CoV-2 antibody, Extracellular vesicles, Proximity barcoding assay, in situ superRCA, mutant transcript

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Zhao, H., **Wang, M**., Muthelo, P., Löf, L., Sterky, F., Gallini, R., Kumar, N. V., Monsen, T., Nilsson, K., Åberg, M., Kamali-Moghaddam, M., Mei, Y. F., & Landegren, U. (**2022**). Detection of SARS-CoV-2 antibodies in serum and dried blood spot samples of vaccinated individuals using a sensitive homogeneous proximity extension assay. *New Biotechnology*, 72, 139–148. https://doi.org/10.1016/j.nbt.2022.11.004

II. **Wang, M.**, Kamali-Moghaddam, M., Löf, L., Fernandez, M., Codin, R., Sterky, F., Åberg, M., Landegren, U. & Zhao, H. Monitoring SARS-CoV-2 IgA, IgM and IgG antibodies in blood and saliva samples using antibody proximity extension assays (AbPEA) (Submitted)

III. **Wang, M.**, Lu, X., Sinha, T., Nilsson, K., Wu, D., Landegren, U. and Kamali-Moghaddam, M. Surface protein profiles of extracellular vesicles reveal SARS-CoV-2 infection (Manuscript)

IV. **Wang, M.***, Chen, L.*, Micke, P., Strell, C., Landegren, U. Highly selective in situ detection of mutant transcripts by using superRCA and gap-fill probes (Manuscript) **equal contribution*

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Abbreviations

SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
Covid-19	Coronavirus disease 2019
RIA	Radioimmunoassay
ELISA	Enzyme-linked immunosorbent assay
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
RCA	Rolling-circle amplification
RCP	Rolling-circle amplification product
PLA	Proximity ligation assay
PEA	Proximity extension assay
PBA	Proximity barcoding assay
FISH	Fluorescence in situ hybridization
sRCA	Super rolling circle amplification
DBS	Dried blood spots
DSS	Dried saliva spots
cfDNA	Circulating free DNA
KRAS	Kirsten rat sarcoma virus
mRNA	Messenger RNA
ncRNA	Non-coding RNA
Ig	Immunoglobin
CLIA	Chemiluminescence immunoassay
LFIA	Lateral flow immunoassays
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgA	Immunoglobulin A
ADAP	Agglutination-PCR
EV	Extracellular vesicle
PSA	Prostate specific antigen
ISEV	International society of extracellular vesicles
smFISH	Single-molecule FISH
FFPE	Formalin-fixed paraffin-embedded
OLA	Oligonucleotide ligation assay
SNP	Single-nucleotide polymorphism

MIP	Molecular inversion probe
ISS	In situ sequencing
HybISS	Hybridization-based in situ sequencing
ROI	Region of interest
NAAT	Nucleic acid amplification test
RT-PCR	Reverse transcription polymerase chain reaction
RT-LAMP	Loop-mediated isothermal amplification-based assay
CT	Computed tomography
RdRp	RNA-dependent RNA polymerase gene

Introduction

Advanced molecular diagnostics uses in vitro biological assays to detect nucleic acids or proteins even in low concentrations across samples, allowing for the identification of biomarkers, monitoring the course of the disease over time, and selection of appropriate therapy. Over a period, several molecular diagnostic tools have been developed, modified, applied and commercialized by our group for analysis of nucleic acids and proteins in situ and in solution. Before starting my PhD, I developed a keen interest in improving methods for molecular analyses. As my PhD research coincided with the outbreak of the highly transmissible and pathogenic SARS-CoV-2 infection, the majority of the work included in this thesis was using samples relevant to Covid-19.

According to immunoassay development history, traditional assays for sensitive protein detection start from the immobilization of antibodies to capture target labeled proteins in solutions, followed by radioactive detection, using radioimmunoassays (RIA) (Yalow & Berson, 1959). Alternatively, samples immobilized on solid phases are detected by labeled antibodies (Figure 1A, 1B). In contrast to assays that require immobilization or labeling of samples to be interrogated, sandwich immunoassays employ immobilized antibodies to capture target proteins from samples, which are then recognized by labeled antibodies. Furthermore, sandwich assays provide higher specificity and sensitivity than those relying only on a single affinity reaction since pairs of antibodies must recognize each target protein simultaneously (Figure 1C).

In 1971, Peter Perlmann and Eva Engvall first published an enzyme-linked immunosorbent assay (ELISA) (Engvall & Perlmann, 1971). ELISA replaced radioactive labels with alkaline phosphatase for protein detection, which avoided the potential health issues of using radioactive substances and ELISA performed as well as radioimmunoassay (RIA) in terms of analytical performance. Over time, ELISA has further developed into different formats, employing chromogenic reporters, chemiluminescence, electrochemiluminescence etc.

The utilization of these chromogenic or fluorophore reporters as readouts leads to several limitations for ELISA. There remain issues of low sensitivity, high background, cross-reactivity with non-specificity, limited multiplexing abilities, etc. At this point, I'm going to explore about another category of immunoassays, which is called DNA-assisted immunoassays. DNA-assisted immunoassay takes advantage of the specificities of antibodies in immunoassays as well as the amplification and detection capabilities of DNA-based

methods. The DNA-assisted immunoassays benefit from many of the advantages of DNA, including its specificity of DNA hybridization, the flexibility of DNA ligation and other enzymatic reactions, as well as its capability to store a large amount of information that can be barcoded and distinguished. Most importantly, nucleic acids can be easily amplified to detectable levels and analyzed using polymerase chain reaction (PCR) or rolling-circle amplification (RCA), as well as microarray hybridization and increasingly high-throughput sequencing techniques (Figure 1D, 1G). It is possible to eliminate background noise caused by any other factors other than detection probes, such as media autofluorescence, which limits fluorescence detection, or background absorbance. Therefore, these achieve high sensitivity and specificity detection via detection of the conjugated DNA strands. Besides, nucleic acid reporter molecules have an excellent capacity to carry information, making it convenient to barcoding for multiplex measurements without cross-activity and elevated background.

The homogeneous proximity ligation assay (PLA) developed in our lab, further modified into proximity extension assays (PEA) and commercialized by Olink Proteomics, detects target proteins by using pairs of oligonucleotide-modified antibodies (Figure 1E, 1F). In preparation of proximity assays, pairs of DNA oligonucleotides are conjugated to each antibody. Once oligonucleotides are brought sufficiently close by having their conjugated antibodies binding to the same target proteins, they can be joined by DNA ligation with PLA, templated with connector oligonucleotides, or extended with PEA. The DNA polymerization or extension reactions provide amplifiable DNA reporters, which can be amplified and quantified by real-time PCR. Alternatively, circularized ligation products can be amplified by rolling-circle amplification (RCA) followed by microscopy quantification for sensitive, localized readout. In addition to form the amplifiable template, the mechanism of requiring two affinity probes to agree on a target provides extra sensitivity and reduces cross-reactivity.

In papers I and II, PEA was for the first time modified to measure specific antibody responses, rather than protein levels as in the standard PEA. We call the method AbPEA and the technique was used to sensitively measure antibody responses to the spike protein or the nucleocapsid of SARS-CoV-2. In paper I AbPEA was demonstrated to offer high specificity, sensitivity and broad dynamic range, with multiplexing potential, offering performance similar to that of other methods for antibody measurements, such as the commercial Abbott (IL, USA) Architect and Meso scale discovery (MD, USA; MSD). Additionally, dried spot test is a bio-sampling technique in which samples are dried and stored on filter paper. We demonstrated utilization of dried blood spots (DBS) and dried saliva spots (DSS) in paper I and paper II, respectively, which further establish that our approach has great potential for large-scale screening and biobanking.

The DNA-assisted immunoassay converts protein identities to DNA sequences for protein detection by using affinity probes with conjugated amplifiable oligonucleotides. PEA and PLA are practical for detecting large numbers of individual protein molecules. However, it is commonly combinations of proteins that are responsible for the pathogenesis in diseases. Therefore, it's worthwhile to explore protein complexes. The proximity barcoding assay (PBA) was originally developed for detection of protein complexes and further validated by profiling 38 surface proteins on individual exosomes (Wu et al., 2019). At present, the technique allows analyses of the presence of even more proteins of large numbers of individual extracellular vesicles from body fluids. In Paper III, we investigated how the protein composition of extracellular vesicles differed among blood samples collected from healthy individual or ones with either mild or severe COVID-19, demonstrating interesting differences.

Last but not the least, DNA-assisted approaches are not only applied to report and identify proteins, but they are even more widely used for detection of nucleic acids. In this thesis I will only cover methods for genotyping of individual RNA in situ. There are several methods used to detect single mRNA molecules in situ, involving hybridization-based fluorescence in situ hybridization (FISH), in situ capture and padlock probe-based approaches. The method I will introduce in paper IV is a padlock probe-based assay. Over the years, padlock probes have been used to sensitively detect nucleic acids in numerous applications, such as genotyping (Hardenbol et al., 2003; Hardenbol et al., 2005), gene expression analysis (Ericsson et al., 2008) and gene copy number analysis(Goransson et al., 2009). Target-dependent enzymatic ligation converts these highly selective probes into circular molecules provided that they have perfectly hybridized with their target sequence. The circular probes can be in situ amplified via RCA, followed by microscopy visualization. In paper IV, we enhanced padlock-RCA-based RNA genotyping in situ by using another newly developed technology for highly selective detection of DNA or RNA sequence variants, referred to as super RCA (sRCA). In superRCA, first round RCA products including many copies of a sequence of interest are interrogated with selective padlock probes, specific for mutant or wildtype variants, followed by second-round RCA. The superRCA method extremely accurately distinguishes sequence variants in situ, because each sequence variant is present in hundreds of localized copies, which avoids any erroneous genotyping. This application provides highly efficient and selective detection of mutant transcripts in a manner that can be clearly resolved from any nonspecific background.

This thesis covers the improvement, modification and application of several molecular techniques previously invented by our group for measuring antigen-specific antibodies (Paper I and II), extracellular vesicles (Paper III) and mutant RNA in situ (Paper IV). These molecular tools can have great value for scientific research and may in the future be applied also for medical diagnosis in patient care.

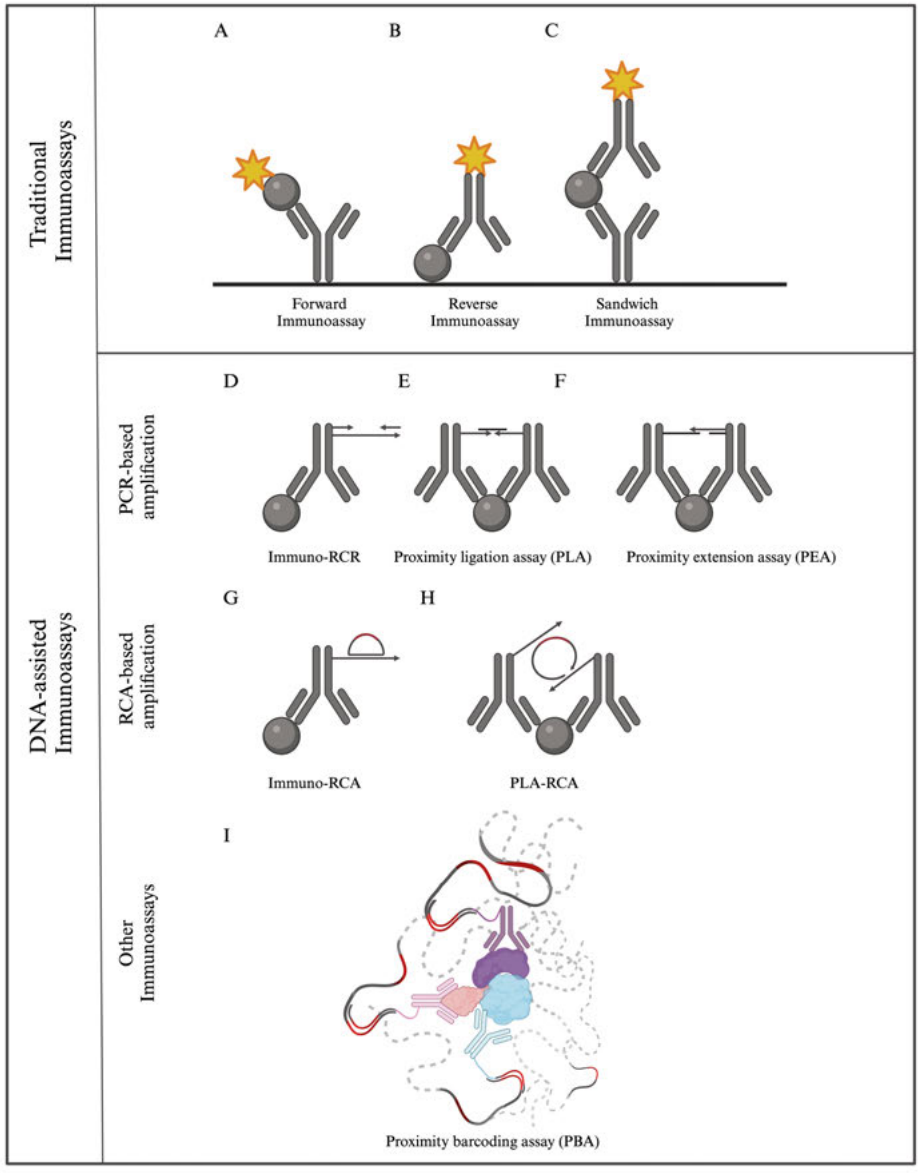

Figure 1. Schematic overview of several immunoassay formats (Created with BioRender.com)

(A) In forward immunoassays, antibodies that have been immobilized capture target proteins from samples that are labeled with detectable moieties (yellow).

(B) In reverse immunoassays, labeled antibodies are used to detect samples immobilized on solid phases.

(C) The sandwich immunoassays use two antibodies, one immobilized for capture and another one was labeled for detection.

(D) In immuno-PCR, amplifiable DNA molecules are directly conjugated to antibodies.

(E) In proximity ligation assays (PLA), two antibody-conjugated oligonucleotides are joined together by DNA ligase if the antibodies bind to the same proteins, followed by PCR amplification.

(F) In proximity extension assays (PEA), DNA strands are extended when pairs or oligonucleotides-conjugated antibodies have bound the same target protein, followed by amplification.

(G) For immuno-RCA, primer oligonucleotides are conjugated to antibodies. Circular DNA templates are introduced into the reactions and hybridized to enable localized amplification through a rolling-circle replication reaction in immuno-PCR.

(H) In RCA-based PLA, circular DNA strands can form only when two oligonucleotide-antibody conjugates bind to the same protein, followed by RCA.

(I) In the proximity barcoding assay (PBA), every protein in the same complex is barcoded with the same DNA tag by copying a unique, repeated sequence in a nearby RCA product, followed by PCR amplification and sequencing.

Diagnostic samples

There is a variety of diagnostic samples that may be collected for analysis, including blood, saliva, excreta, body tissues, tissue fluids, and so on. Various approaches can be used to obtain diagnostic samples from different parts of human body. Most blood samples are collected by inserting a needle into a vein in the arm or, more rarely, by pricking a finger. Saliva samples can be collected from the mouth. Furthermore, urine, stool, sputum, tears and vomitus can be collected directly as they leave the body by direct collection at the time of exit. Selection of appropriate samples is important both in clinical diagnosis and for scientific research. First, samples should contain target molecules or substances at detectable levels, and the resultant signal should be clearly distinguishable from background or control levels. Next, there should be adaptable assays available for the samples that have sufficient reproducibility, sensitivity and specificity for the analyses to be undertaken. Furthermore, several other factors should be considered based on the purpose and diagnosis scenario, such as simplifying procedures, lowering costs and increasing throughput.

In this thesis, I will only cover the types of samples that I have used -blood and saliva - and in particular discuss what advantages they possess when they are stored on filter papers in a dry state, and tissue specimens. I will also discuss the human tissue section samples used in the last paper.

Blood specimens

In general, blood is an ideal source of samples for a wide variety of diagnostic tests, since it can be easily accessed and the altered levels of molecules released into blood may be indicative of disease processes at any location in the body. Blood samples (several milliliters) are usually obtained through venipuncture by a trained individual and followed by centrifugation and plasma extraction, which is hard to fully standardize. By educating samplers, consistency can be increased in the sampling procedure, however repeated practice is necessary to maintain a high standard (Bolenius et al., 2013). The traditional method for collecting venous blood samples also has the disadvantage of involving larger volumes (2.5-10 ml) than what is often necessary for the intended analysis. Furthermore, the samples are usually stored in tubes frozen at -80°C, occupying substantial space in energy-consuming freezers.

Capillary blood samples are often used to collect small blood samples through a finger stick using a lancet. Blood samples taken from capillaries are less invasive than those from veins, and the patients can perform a finger stick themselves at home. Despite these attractive features, capillary samples have some limitations, for instance, blood clotting and hemolysis can be more difficult to prevent in capillary samples than in venous samples. Moreover,

squeezing the fingers to enhance blood flow may contribute to extracellular fluid that can lead to hemolysis and distorted sample composition.

Dried blood spots (DBS) are typically generated from capillary blood, which provides several advantages over conventional blood sampling. DBS sampling involves collecting a droplet of blood and storing in a dried state on a filter paper, usually obtained by lancing a finger. DBS offer the advantages of easy sampling, minimal invasiveness for patients and convenience of transport via regular mail delivery, which can all increase the efficiency of large-scale screening. In comparison to wet blood storage, DBS have a compact format and admits a higher potential storage temperature, which reduces the cost of sample storage in biobanks for a long period of time (Lim, 2018; Zakaria, Allen, Koplin, Roche, & Greaves, 2016). Previous studies have also demonstrated that antibody levels detected in DBS samples correlated well with traditional serum or plasma sample (Brinc et al., 2021; Morley et al., 2020). Thus, DBS collection is superior to wet blood due to the ease of non-invasive collection, and it may in the future replace wet samples for clinical diagnosis.

Based on the above benefits, we monitored in paper I changes of antibody levels during the course of two consecutive mRNA vaccinations against SARS-CoV-2 by using serially self-collected DBS (Figure 2).

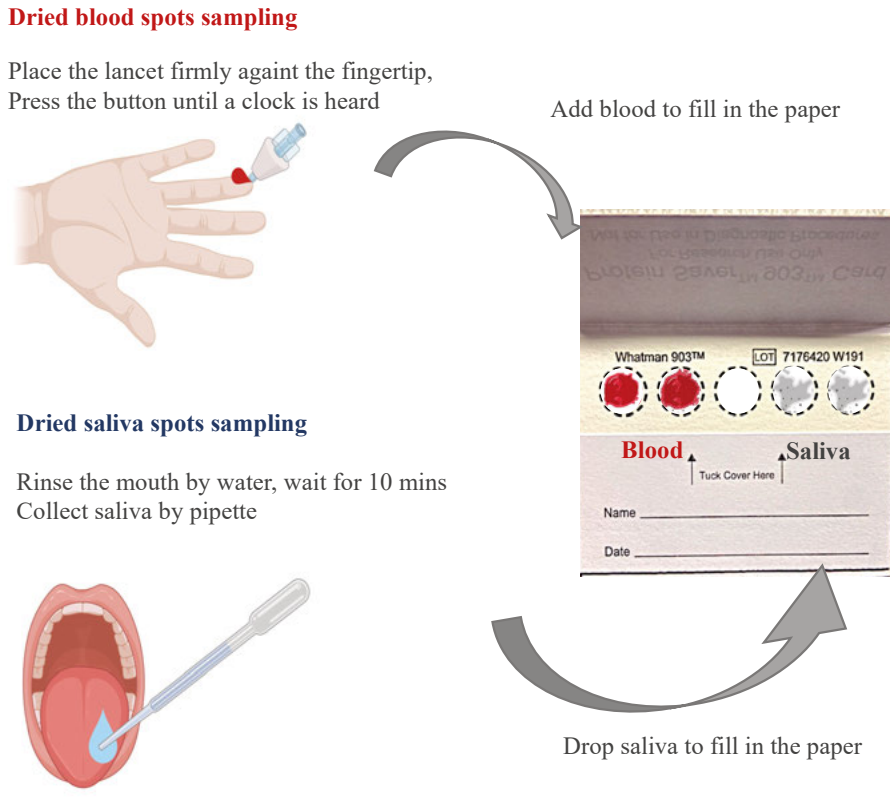

Figure 2. Dried blood spots and dried saliva spots sampling procedures (Created with BioRender.com)

Saliva specimens

Since fingertip blood collection may still be considered somewhat invasive and uncomfortable, saliva is another useful biological fluid that can be used for prognosis, laboratory diagnosis and patient monitoring for various diseases. In addition, it can be easily collected without specialized training, non-invasively without any discomfort as dried saliva spots (DSS) and stored easily. Saliva contains specific biological markers that can make it an ideal sample for detection of disease (Malamud, 2011). Currently, salivary diagnostics is becoming increasingly important for diagnosing and monitoring progress of disease, and as a basis for making clinical decisions regarding patient care. According to a recent review, salivary antibody levels can be used to detect responses to a number of viral diseases and other infectious diseases or to monitor the immune status of infected individuals (Kaufman & Lamster, 2002). Besides, saliva samples have lower transmission risk comparing to blood sampling. Saliva collection presents several advantages over blood as listed above, and it should be considered as an alternative to blood as

diagnostic matrix. This is illustrated by the strong correlation between antibody responses measured in saliva or in blood during acute infection and recovery (Isho et al., 2020).

For DSS sampling, small volumes of saliva are collected and stored on filter paper and remain stable for long periods of time, reducing the difficulty of transportation and storage (Han et al., 2022). One possible disadvantage of DSS is limited sample availability for analysis, which requires a detection method with high sensitivity. However, most techniques for analyzing dried samples have been validated on DBS, which means that more effort must be spent on validating technologies for DSS analysis.

In paper II, DSS are used for antibody analysis. We applied AbPEA (developed in paper I) to evaluate SARS-CoV-2 salivary antibodies stored on filter paper.

Tissue specimens

Biological tissue specimens are commonly used sample types in biology and medical research. Samples of tissue can be obtained from various sources, such as tissue biopsies obtained during surgical resections or at autopsies. A biopsy or endoscopic procedure can provide detailed information about a diseased tissue with minimally invasive procedures. A surgical specimen can provide a larger representation of the affected tissues, allowing for further research on tissue morphology and histology. However, surgical operation itself provide extra difficulty for tissue collection with higher risk. The collection of tissues mentioned above must follow strict legal and ethical guidelines with the involvement of a trained pathologist in the procedure (Campbell et al., 2012).

The storage condition of tissue samples should be considered to preserve the quality of samples and avoid degradation of valuable molecules. A properly stored sample is particularly important if it is going to be used for RNA analysis such as in paper IV, since RNA degrades quickly if it is not stored properly. Tissue samples should be stored and transported at the appropriate temperature. Tissue samples can be preserved with commonly used methods such as cryopreservation or formalin fixation and paraffin embedding to preserve their quality and integrity (Annaratone et al., 2021).

Currently, tissue samples play a significant role in identifying genes, transcripts, proteins and signaling pathway within cancer and other medical research. Valuable information from human tissues can be used to predict cancer in earlier stages and develop personalized therapeutic regimens with fewer side effects (Hamburg & Collins, 2010). In addition, pharmaceutical companies apply molecular analysis, tissue microarrays, and immunohistochemistry analysis methods on tissue samples to uncover disease mechanisms or biomarkers associated with specific types of diseases (Vaught, Rogers, Carolin, & Compton, 2011). Compared to blood and other body fluids, tissue samples

provide extensive information and directly reflect the stage of the diseases. However, it can be difficult to obtain and process high-quality tissue samples, techniques for spatial analysis can also be challenging.

In paper IV, we utilized fresh frozen tissues with KRAS mutation to validate our newly developed in situ method for highly selective mutant transcripts detection.

Valuable biomarkers in samples

In commonly used diagnostic samples such as blood, a number of circulating factors can be detected, including cell-free DNA, circulating RNA, proteins, antibodies, circulating cells or cellular debris, exosomes, etc (Michela, 2021). Some of the circulating factors can serve as leakage biomarkers for diseases. For instance, circulating free DNA (cfDNA) in peripheral blood was first discovered in 1940s by Mandel and Métais (Mandel & Metais, 1948). The cfDNA fragments are typically double stranded DNA molecules in multiples of 166 base pairs. Recently, there has been an increased interest in using DNA derived from plasma or other biological fluids for clinical diagnosis, due to its minimal invasiveness and clinical significance (Mader & Pantel, 2017). By using cfDNA, it has proven possible to detect trisomies, subchromosomal aberrations, and even monogenic disorders with simple blood draws. Oncologists and other researchers have continued to develop reliable biomarkers using cfDNA and it also allows testing fetuses by drawing blood from the pregnant mothers (Szilagyi et al., 2020). However, blood contains very low amounts of cfDNA, only a small amount of which derives from the fetus or the diseased tissues. This requires analysis methods that are both highly sensitive and specific.

A wide variety of RNA molecules are present in human serum and plasma, including miRNAs and various other non-coding RNA (ncRNA) molecules, as well as protein-coding messenger RNAs (mRNA). Protein-coding messenger RNA (mRNA) was the first and most widely studied type of RNA, serving as cancer biomarker associated with pathology. For example, upregulation of mRNA in peripheral blood has been detected from patients with breast cancer (Gal et al., 2001), lung cancer (Fleischhacker et al., 2001), prostate cancer (Chu et al., 2004) etc. Besides, other types of RNAs with regulatory functions also have the potential to serve as biomarkers to classify different cancer diseases.

Specific proteins and antibodies are both common biomolecules in the circulation, offering valuable information regarding the progression of the disease and the outcome of therapies. A number of proteins found in blood plasma have been used as cancer biomarkers for many years, and there is a large and rapidly growing literature about research focusing on the search for protein markers. For instance, prostate specific antigen (PSA) is a biomarker protein, which can be used to detect prostate cancer or monitor recurrence after tumor removal surgery. Elevated levels of PSA in the blood may indicate the presence of prostate cancer since PSA is normally present in the blood of men at very low levels. While the current state of protein liquid biopsy is far from satisfactory, a combination of proteins and DNA biomarkers may provide a promising future (Cohen et al., 2017).

Many infectious disease diagnostics are based on detection of antigen-antibody complexes, and by testing for antibodies, we are able to determine

information about patients' disease history, including exposures to infections and on-going infections. In addition, it is helpful for identifying people who may be asymptomatic but nonetheless potentially capable of transmitting a disease. In paper I and project II, detection of SARS-CoV-2 antibodies provides valuable information about the immune status of vaccinated and infected individuals.

Exosomes, microvesicles, and apoptotic bodies are the three types of extracellular vesicles classified by the international society of extracellular vesicles (ISEV). Small membrane-derived particles known as exosomes and microvesicles are released by most cells. They are thought to play a significant role in cell signalling and immune responses, as well as perhaps promoting tumor cell proliferation (Hoshino et al., 2015). EVs can be found in nearly all body fluids, and they are therefore considered potential candidates for analysis by liquid biopsy. Surface proteins of EVs in biofluids exhibit high heterogeneity in their molecular composition, as they originate from different sources of tissues (Poliakov, Spilman, Dokland, Amling, & Mobley, 2009). In paper III, we used proximity barcoding assays (PBA) to compare surface protein composition of EVs between SARS-CoV-2-infected individuals and healthy individuals.

Advanced molecular techniques for diagnosis

Methods for antibody detection

As a special class of protein, antibodies (Ab), also known as immunoglobulins (Ig), help the immune system recognize and neutralize foreign objects such as bacteria and viruses (Litman et al., 1993). There are five isotypes or classes of antibodies: IgA, IgD, IgE, IgG, and IgM, which differ by their biological structures and properties, functions, working locations and how they deal with antigens. In this thesis, I will not discuss the structures, functions and classification of antibodies, as I will mainly focus on methods for detection of total antibodies or isotypes of IgG, IgM and IgA.

Antibody detection has been widely used and plays an important role in the diagnosis and monitor of various diseases. It's worth mentioning that antibody detection techniques are significant for identification of current and past infection disease, as well as revealing immune status of infected or vaccinated individuals against certain pathogens.

Several approaches are commonly used for antibody detection. Laboratory-based methods for detection of antibodies, such as ELISAs, chemiluminescence immunoassays (CLIAs) and lateral flow immunoassays (LFIAs), serving as diagnostic tools for the purpose of improving access to diagnosis of infection or autoimmune diseases, providing useful information about the immune status of sampled individuals as well as screening asymptomatic individuals to stem transmission (Makoah et al., 2021).

ELISAs are generally considered the gold standard of immunoassays in clinical laboratories because their capacity to sensitively detect and quantify a wide range of substances, including antibodies, proteins and hormones. ELISAs can be used to detect antigen-specific antibodies from patients by using immobilized antigen on the ELISA plate to capture antibodies from patient samples (Del-Rei et al., 2019). The assays generally rely on the principle of forming complexes of immobilized antigen–antibody–secondary antibody binding, The captured patient antibodies are detected using enzyme-conjugated detection secondary antibodies specific for antibody isotypes of interest (e.g., IgG, IgM, etc.). Following the interaction between the conjugated enzyme and a suitable substrate, a colorimetric change occurs which can be quantified and correlated to antibody concentrations (Mohit, Elham et al. 2021). ELISAs are developed by comparing different antigens and antibody isotypes in order to achieve the highest level of sensitivity and specificity

(Espejo et al., 2020). The advantages of ELISAs include their relatively low cost and the ability to detect both antibody titers and isotypes in common samples. Weak signals, uncertain cut-offs between positive and negative samples (high possibility of false positive/negative), long resulting time, complex and labor-intensive workflow, and limited dynamic ranges are problems with some ELISAs (Isho et al., 2020; Mekonnen et al., 2021; Sakamoto et al., 2018).

Lateral flow immunoassays (LFIAs), are paper-based devices that are based on the principle of migrating liquid samples containing analyte of interest through capillary action, passing through zones in the paper strips where the analyte can interact with both solution-phase and immobilized reagents. Initially, the liquid sample containing the target analyte is placed on an adsorbent sample pad, at one end of a strip. The analytes present in samples then dissolve buffer salts and surfactants in sample pads. Next, samples are moved by capillary action to the conjugate release pad, which contains conjugated antibodies or antigen conjugated to colored or fluorescent particles. Then the conjugated antibody/antigen and target migrate towards detection zones in the strip, which consists of lines of immobilized capture antibodies (anti-human IgG and IgM antibodies). At the end of the strip, an absorbent pad absorbs excess reagents, maintains liquid flow, and prevents backflow (Koczula & Gallotta, 2016). Finally, test line responses indicate the presence of the target analyst in the sample, whereas control line responses serve to demonstrate that the test is valid. The results of LFIAs are displayed within 5 to 30 minutes and the majority of rapid point-of-care (POC) immunoassays utilize lateral flow immunoassays (LFIAs) (Li et al., 2020). Meanwhile, considering that LFIA devices are cheap to manufacture, store, and distribute, they present a number of advantages for antibody screening in large populations.

Luminescence is a detection modality that occurs when an electron transits from an excited state to the ground state, in the process emitting visible or near-visible light. Chemiluminescence is the process of emitting light by using chemical reactions as the source of energy for the generation of electronic excitation (Cinquanta, Fontana, & Bizzaro, 2017). The chemiluminescence immunoassay (CLIA) combines chemiluminescence with immunochemical reactions (Cinquanta et al., 2017). This technique takes advantage of the specificity of the immune response as well as the sensitivity of the luminescence reaction with high signal-to-noise ratios. The method can be used automatically to quickly analyze large numbers of samples on immunochemical analyzers (Wan, Li, Wang, Li, & Liao, 2020). CLIAs are similar to ELISAs, but simpler to perform. Since they have shorter incubation steps and do not require a reagent to stop the enzyme reaction, they are able to provide a greater throughput with increased sensitivity and dynamic range.

Luminex assays are bead-based immunoassays that allows for quantifying multiple analytes simultaneously. In these assays, beads or microspheres are internally dyed with red and infrared fluorophores of differing intensities,

which corresponding to a distinct spectral signature or different classes of beads. Then each set of beads is coated with unique capture antigens for specific antibodies, followed by blocking and washing (Dobano et al., 2021). The captured antibodies from a sample are detected using a specific biotinylated detection antibody plus streptavidin-conjugated phycoerythrin (SA-PE). Finally, two lasers are used in the Luminex® fluorocytometer, one excites the fluorochromes in the bead and the other excites the PE bound to the detection antibodies. Therefore, the first readout reveals the bead-specific signal, as well as the antigen-specificity of bound antibodies, while the other readout indicates to what extent antibodies in the sample have bound to the antigens attached to beads (Tait, 2016). Luminex technology presents several advantages as follows: high sensitivity, wide dynamic range, multiplexing and high throughput. However, Luminex requires specialized equipment and training. The results of multiplexed assays can be inaccurate due to cross-reactivity between analytes.

In paper I and paper II, we sensitively measured antibody responses to the spike or nucleocapsid proteins of SARS-CoV-2 in blood and saliva. It's interesting to know how well the antibody detection methods mentioned above perform in SARS-CoV-2 antibody analysis when using blood and saliva samples in both wet and dry form. The common techniques above have been widely used in COVID-19 serological tests. For instance, a well-validated Wantai ELISA kit with 79% sensitivity was used to measure total antibodies against the the spike protein of SARS-CoV-2, including IgM, IgG, and IgA (Nilsson et al., 2021). In samples collected $\geqslant$4 days after positive RT-PCR, Euroimmun anti-SARS-CoV-2 ELISA demonstrated 90.5 and 100% agreement for IgA and IgG detection, respectively. The sensitivity and specificity for IgA detection by using Euroimmun anti-SARS-CoV-2 ELISA is good while it is excellent for IgG (Beavis et al., 2020). Modified ELISAs have also been validated for antibody detection in several dried form samples. By applying Euroimmun ELISA, 3 mm punches of DBS reached 96.8% and 81.3% positive and negative agreement compared to serum, respectively (Turgeon et al., 2021). Comparing plasma with DBS by using Siemens SARS-CoV-2 total antibody assay, there was a 100% and 94.4% agreement for negative and positive patients, respectively (Omosule et al., 2023). The relative sensitivity and specificity of DBS samples in detecting antibodies against S glycoprotein were 98.11% and 100%, respectively (Morley et al., 2020). While most serological tests of dry samples use blood, the GSP/DELFIA technique detected IgG in DSS with 85.3% sensitivity and 65.7% specificity to differentiate sample donors having received two vaccinations from all other exposure types (Lahdentausta et al., 2022). The assays require at least 3 to 6 mm punches from dried spots and require 1 hour elution time and they offer no certain cut-off (Lahdentausta et al., 2022; Morley et al., 2020; Omosule et al., 2023; Turgeon et al., 2021).

One of the most widely used CLIAs for Covid-19 is the Abbott Architect SARS-CoV-2 IgG test. This assay uses paramagnetic beads coated with recombinant viral antigen, enzyme-labeled antibodies and a luminescent substrate for automated analysis. Using Abbott Architect IgG test, one commonly used CLIA, the sensitivity for detection of SARS-CoV-2 IgG reached 94% to 100% after 14 days post symptom onset with specificity between 99% and 100% (Bryan et al., 2020; Theel, Harring, Hilgart, & Granger, 2020). While the assay is relatively sensitive, there is a considerable rate of false positive results, as well as a limited dynamic range. The level of IgG in patient samples does not correlate well with the time post-infection (Bryan et al., 2020).

All the methods I have described above employ fluorophores or enzymes as reporters directly coupled to antigens for antibody measurement. I will next describe DNA-assisted immunoassays. DNA-assisted techniques that are widely utilized for protein detection can also be applied to antibody detection. In these assays, instead of directly measuring antibodies, nucleic acids coupled to antibodies serve as DNA reporter for signal amplification. Two commonly used amplification strategies for DNA, polymerase chain reaction (PCR) and rolling circle amplification (RCA), can be used to amplify the DNA tag or sequences associated with antigen-antibody complexes, which increases the detection limit of traditional immunoassays. Additionally, DNA-assisted assays can increase detection specificity, reduce background noise and offer a potential to be multiplexed.

As an innovative technique, proximity-based DNA-assisted immunoassays improve immunoassay sensitivity and specificity by taking advantage of the proximity design. In contrast to standard immunoassays, the two homogenous proximity assays: proximity extension assay (PEA) and proximity ligation assay (PLA) have similarities and differences (Fredriksson et al., 2002). PEA and PLA both require the recognition of targets by pairs of affinity reagents, just as sandwich ELISAs do, but they do not involve solid-phases for capture and no washing is required and the antibody pairs are modified by conjugation to oligonucleotides. Quantification of proteins is accurate even at low concentrations and over wide dynamic ranges. For multiplex assays that may involve a hundred or more targets, generally only single microliters of sample are required (Assarsson et al., 2014). In the assays, pairs of oligonucleotide-conjugated antibodies that are brought together by binding to the same target protein molecules in a sample. This induced proximity allows the conjugated oligonucleotides to generate amplifiable DNA strands, either via DNA polymerization (PEA) or ligation (PLA). Then PCR or DNA sequencing reactions use these amplicons as surrogate markers for target proteins. PEAs can be used for multiplex protein detection, while we have also demonstrated that the assays can also be applied for detecting antibodies directed against specific antigens by conjugating barcoded oligonucleotides to pairs of antigens instead of pairs of target-specific antibodies. In the assays, patient antibodies in the sample bring pairs of antigen molecules together, resulting in the production

of amplicons serving as surrogate markers for antigen-specific antibodies. A homogenous PCR-based assay, AbPEA, was validated in paper I as a sensitivity and convenience test for detection of antibody responses to SARS-CoV-2 after infection or kinetics of antibody responses after vaccination (Zhao et al., 2022) (Figure 3). Agglutination-PCR (ADAP) is a technique similar to AbPEA that detects antibodies against SARS-CoV-2 in serum and plasma samples as well as DBS samples (Karp, Cuda, et al., 2020; Karp, Danh, et al., 2020).

However, ADAP uses 4 µl of neat serum, and DBS need to be eluted and concentrated for analysis. As validated in paper I, AbPEA only requires 1 µl of neat serum or 1.2 mm disk cut from a DBS with high sensitivity and specificity. Besides, AbPEA has several other advantages such as simple and effective procedure, broad dynamic ranges, no need for secondary antibodies, solid phase capture or elution steps, suitable for multiplexing etc. In paper I, AbPEA was shown to be highly specific and sensitive for solution phase antibody detection in serum and DBS samples, without need for secondary antibodies and elution steps, and with a potential for multiplex and high throughput analysis. In paper II, antibody levels towards SARS-CoV-2 were measured in dried saliva samples that can be collected noninvasively, and a modification of the AbPEA technique was shown to also permit measurement of class-specific antibody responses.

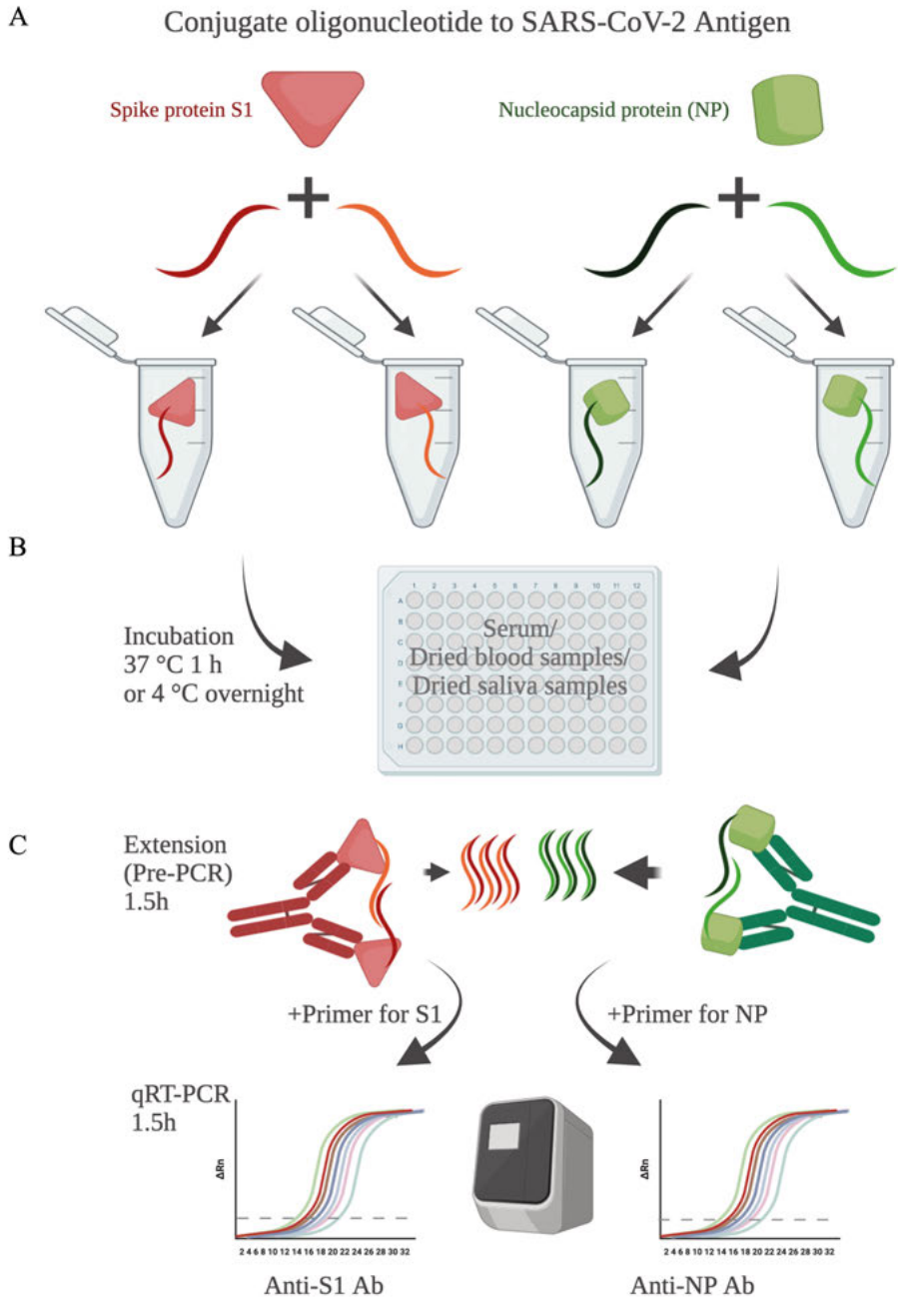

Figure 3. Schematic illustration of AbPEA for antibody detection (Created with BioRender.com)

(A) Recombinant S1 or NP antigens were conjugated to two pairs of oligonucleotides. Each pair of oligonucleotides contains a specific barcode, a mutually complementary region, and a target sequence for a molecular beacon for real-time PCR detection. (B) Samples were incubated with oligonucleotide-conjugated protein probes. When pairs

of oligonucleotide-conjugated antigen molecules are bound to the same antibodies, the complementary oligonucleotides are hybridized to each other, (C) allowing DNA extension by a DNA polymerase with incorporation of barcodes from both probes into DNA extension products. These DNA extension products are then amplified with universal primers to generate sufficient amounts of amplicons. Finally, antigen-specific amplicons are quantified by real-time PCR using specific primers and molecular beacons to record at which PCR cycle (Ct) the recorded fluorescence exceeds a threshold value.

Methods for extracellular vesicles (EVs)

Extracellular vesicles (EVs) are capable of transporting and delivering multiple functional biomolecules, including DNA, microRNAs, messenger RNA (mRNA), long non-coding RNA and surface and internal proteins - cargo that has been suggested to serve as biomarkers in many previous studies. Exosomal biomolecules could therefore have a high diagnostic value. The molecular compositions of EVs are highly heterogeneous due to their diverse sources of origin, and they contain components of the cells and tissues from which they originate (Poliakov et al., 2009). By differentiating EVs according to their unique surface proteins we can identify EVs unique to the patient and help identify the pathological features present in their original cells or tissues (Tavoosidana et al., 2011). Most commonly total EVs from samples are extracted and total protein, DNA and RNA in the EVs are quantified. However, we can better understand EV heterogeneity of EVs originating from diseased cells by using techniques to characterize single EV. I will dedicate this section to review techniques to analyze single EV, since we profiled surface proteins of individual EVs by using a novel proximity barcoding assays (PBA) in paper III, and recent years have seen the emergence of several other new technologies for single-EV analysis, worthy of our attention.

For instance, imaging flow cytometry overcomes limitations in traditional flow cytometry by enhancing fluorescence to detect vesicles smaller than 500 nanometers (Erdbrugger et al., 2014). ExoELISA is an immunoassay for digital quantification of EVs based on droplet-based single-EV counting. This enzyme-linked immunoassay (droplet digital ExoELISA) counts single EVs and can be used to obtain an unprecedented level of accuracy in counting cancer-specific EVs (Liu et al., 2018). An array of nanoholes in nanoplasmonic sensors is used to separate single EV first through the use of specific recognition antibodies, followed by the profiling of proteins through the use of detection antibodies (Im et al., 2014). The multiple recognition proximity ligation assay (4PLA) can be used to identify EVs that originate from the prostate through the use of a capture antibody and four probe antibodies with high sensitivity and specificity (Tavoosidana et al., 2011).

The above methods all have limitations, particularly in terms of throughput and because of their inability to detect multiple surface proteins because of the

limited number of fluorescent colors that can be distinguished. By applying proximity barcode assays (PBA), it is possible to detect surface protein composition of individual EV in high throughput. In PBA, individual EVs are barcoded using micrometer-sized single-stranded DNA clusters containing hundreds of copies of a unique DNA motifs, prepared by RCA. In paper III, we conjugated antibodies directed against 181 surface proteins with unique oligonucleotides for each antibody specificity (antibody tags). After immobilizing EVs in microtiter wells the antibody pool was added, followed by washes. Next, the RCA products were added and washed, and antibody-conjugated oligonucleotides were the extended to copy unique tags on nearby RCA products. In this manner, oligonucleotides on antibodies having bound the same EV acquired the same specific code from a particular RCA products, serving as a tag for that EV (EV tag). Next all antibody-conjugated oligonucleotides that were extended by incorporating and EV tag were amplified by PCR amplification, in the process also incorporating a sequence element that serves as a sample-specific DNA code (sample tags). Finally, the sequence information of individual amplicons was determined by next-generation sequencing, revealing combinations of antibody tags, EV tags and sample tags. This technique thus identifies combinations of proteins on individual EV, for high throughput surface proteomic analysis of single EVs. The simple experimental procedure provides the possibility to simultaneously analyze large numbers of EVs in many samples in each experiment, with no need for EV purification.

Methods for spatial RNA detection

In situ techniques are used to reveal and also quantify spatial information about specific DNA, RNA or proteins, which is important for understanding regulation of cells, identify biomarkers and monitor the progress of disease, evaluate therapy and etc. In this thesis, we cover a method for localized detection of mutant transcripts in paper IV, so I will only review and discuss several principal approaches for in situ RNA profiling.

In situ hybridization (ISH)

Fluorescence in situ hybridization (FISH) is one of the most commonly used and convincing techniques for detecting and localizing the presence of specific DNA or RNA, which can further define gene expression pattern and diagnose certain diseases. In FISH, DNA or RNA probes coupled to fluorescent reporter molecules are annealed to specific target sequences of sample. As early as 1980, fluorescently-labeled RNA probes were firstly reported to detect specific DNA sequences via fluorescent in situ hybridization (FISH) (Bauman, Wiegant, Borst, & van Duijn, 1980). FISH has later been developed and applied for visualization and quantification of RNA in situ, referred to as RNA-FISH (Levsky, Shenoy, Pezo, & Singer, 2002; Raj, van den Bogaard, Rifkin, van Oudenaarden, & Tyagi, 2008; Singer & Ward, 1982).

Single-molecule FISH (smFISH) was designed for sensitive detection and quantification of individual mRNA transcripts (Femino, Fay, Fogarty, & Singer, 1998). In smFISH, multiple oligonucleotide-probes were labeled with several Cy3 molecules, which were then hybridized to target mRNA to generate fluorescent signals with high intensity. Later, smFISH was modified due to difficulties in synthesizing heavily labeled probes. By using a series of 20-nt oligonucleotide probes, each coupled to a single Alexa 594 fluorophore, individual targeted mRNA molecules could be detected, producing cumulative fluorescence signals with low false-positive rate and using a simple probe synthesis process (Raj et al., 2008).

RNAscope is another in situ hybridization technique that uses several pairs of probes per transcript where each pairs anchors a large detection complex for identification of single mRNA molecules (De Biase et al., 2021; Wang et al., 2012). RNAscope is applicable to formalin-fixed, paraffin-embedded (FFPE) samples, compatible with laboratory workflows, and may be multiplexed. However, even though these approaches provide high sensitivity and efficiently detect single mRNA transcripts, it's remaining challenging to distinguish and identify highly similar sequences such as point mutant transcripts verus wildtype.

In situ capture

Ståhl and others presented an in situ transcriptome visualization approach called "spatial transcriptomics", which allows capturing transcriptional activity with spatial information (Stahl et al., 2016). In this technique, tissues are first permeabilized, and then spatially barcoded reverse transcription primers located in a grid structure bind and capture adjacent mRNAs released from tissue cryosections. The barcoded primer contains oligo (dT) for mRNA capture, a spatial barcode, amplification and sequencing handles, a cleavage site and a unique molecular identifier. Reverse transcription is carried out in situ after probe binding, producing complementary DNA with spatial barcodes corresponding to the position in the grid structure. The cDNA molecules are then released and amplified before a next step where the cDNAs are sequenced with Illumina sequencing. Spatial barcodes in the sequencing data map each mRNA transcript back to the origin in the tissue. Spatial Transcriptomics, a spinout company, commercialized this method and was acquired by 10x Genomics in 2018.

10x Visium is an improved version of the spatial transcriptomics approach, which provides mRNA profiling for tissue classification. This system also employs spotted arrays on the surface of glass slides to capture mRNA, but with more spots, smaller spots size, and more probes per spot. The diameter of each barcoded spot is 55 μm, and the distance between the centers of nearby point is 100 μm. The average amount of mRNA captured per spot ranges from 1 to 10 cells, allowing for practically single-cell resolution. 10x Visium has many advantages: capable of whole transcriptome analysis in situ, unbiased detection of whole transcriptome, nearly single-cell high resolution, compatible with FFPE and fresh-frozen tissue samples. However, RNA capture efficiency remains a major challenge for these methods, especially as resolution increases with smaller capture spot.

Padlock-based in situ sequencing

Over the past years, Mats Nilsson, George Church, and others have been genotyping and sequencing transcripts directly in tissue sections. I will only review padlock-based assay for RNA profiling.

Padlock probes originated three decades ago (M. Nilsson et al., 1994) as a further development of the oligonucleotide ligation assay (OLA) (Landegren, Kaiser, Sanders, & Hood, 1988). Typically, padlock probes are 70-100 nt linear oligonucleotides with target-complementary 5' and 3' ends, referred to as arms, which must hybridize next to each other on the correct sequence for target recognition. Upon hybridizing to their correct targets, the two arms of padlock probe align head to tail, with just a nick between them to be sealed by a DNA ligase enzyme. After padlock circularization, it is possible to amplify the circularized probes with PCR or RCA (Lizardi et al., 1998), which will be

discussed further below. The padlock-RCA products are topologically locked to the target, which makes them resistant to washes leading to reduced non-specific background. The characteristics listed above provide several advantaged for padlock approaches: easy multiplexing with low cross-reactivity rate, low non-specific signals and high selectivity, allowing for distinguishing highly similar sequences such as point mutation and single-nucleotide polymorphism (SNP) (Baner et al., 2003; Hardenbol et al., 2005).

Gap-fill padlock probes are an alternative version of padlock probes in which the target-complementary ends hybridize leaving a gap between them. The gap between to padlock-arms need be filled by either polymerization or using an intermediary oligonucleotide before the probe can be converted to circles by ligation (Akhras et al., 2007; Porreca et al., 2007) A gap-filling approach with a single nucleotide gap has been used for highly multiplexed genotyping using so-called molecular inversion probes (MIP) (Hardenbol et al., 2005). Gap-fill padlock probes leaving a longer gap have been used for sample enrichment before sequencing (Porreca et al., 2007). Another variant on the same theme is the selector probe. This probe is designed to hybridize to both ends of one strand of a DNA restriction fragments, followed by ligated to create a circle (Dahl, Gullberg, Stenberg, Landegren, & Nilsson, 2005; Isaksson et al., 2007).

In situ sequencing (ISS) is a padlock probe-based technique where RCA products of reacted probes are sequenced in situ (Ke et al., 2013). In ISS, mRNA is first reversed transcribed to cDNA in preserved cell lines or tissues and mRNA is then degraded with RNase H. There are two strategies for in situ RNA sequencing. The first approach is gap-targeted sequencing, in which gap-fill probes bind to cDNA by leaving a gap between the ends over the bases that will be targeted for sequencing by ligation (Asp, Bergenstrahle, & Lundeberg, 2020). Next, DNA polymerization is used to fill in the gap to form a DNA circle by ligation. For barcode-targeted sequencing, padlock probes with barcode sequences are hybridized to the target, followed by ligation to form DNA circles. For both strategies, DNA circles are then amplified by RCA, yielding rolling-circle products (RCP). The products, consisting in repeats of the padlock probe sequences, canbe then decoded by sequencing by ligation. By contrast to the approach using padlock probes with barcoded sequences, gap-filling version reveal sequence information for the targeted transcript. A main advantage over in situ hybridization-based assays for ISS is the possibility of distinguishing highly similar sequences such as single nucleotide variations (SNPs). In 2017, this padlock-based in situ RNA sequencing method was commercialized as kits and services by the company Cartana. The company was acquired by 10X Genomics, which is commercializing n automated version of the technique as the Xenium platform (Maino et al., 2019).

The ISS method has a good signal detection and throughput, but low transcript detection efficiency due to several inefficient steps such as cDNA synthesis, padlock probe hybridization and ligation (Ke et al., 2013; Qian et al.,

2020). Although there is no previous work to prove this, insufficient chemical post fixation might also contribute to low efficiency. The reverse transcription step can be bypassed by probing mRNA directly in situ, thereby increasing detection efficiency. In direct RNA targeted approach, RNA may be directly probe and read out with ISS (Lee, Marco Salas, Gyllborg, & Nilsson, 2022), showing fivefold improvement of transcript detection efficiency compared to cDNA-based HybISS (Gyllborg et al., 2020) without decrease of specificity and multiplexing capabilities (Lee et al., 2022). However, a downside of increased detection efficiency is optical crowding, where RCPs can no longer be separately distinguished during combinatorial decoding. Additionally, the enzymes used for RNA templated DNA ligation shows greater tolerance for mismatching substrates, leading to a lower specificity than DNA ligation with cDNA templates (Krzywkowski & Nilsson, 2017). There is no relevant paper proving that direct RNA targeted in situ approach can still be used to distinguish SNPs.

Few previous papers have studied the application of these RNA detection techniques for mutation detection, especially mutation in weakly expressed transcripts, such as KRAS (kirsten rat sarcoma virus) mRNAs. Mutation detection in these transcripts requires optimized assays with high selectivity and specificity, as low frequencies of erroneous genotyping can affect the recorded cellular status, which might lead to wrong guidance for effective treatment. Because of the need for extremely selective typing of mutant transcripts in situ in order to accurately identify even very rare tumor cells in situ we have devised a highly accurate localized genotyping technique, building on the superRCA mechanism in paper IV. SuperRCA allows for highly precise distinction of sequence variant since each genotyped sequence variant is present in hundreds of localized copies, allowing any erroneous genotyping reaction by individual padlock probes to be safely ignored (Chen et al., 2022). To begin with, cDNA synthesis primers for reverse transcription of sequences of interest are added to tissues, which are then crosslinked to ensure proper localization. After reverse transcription, gap-fill padlock probes are added targeting regions of interest (ROI) in reverse transcripts after RNaseH-mediated degradation of the copied mRNA, and the probes are sealed into DNA circles. Next, RCA is initiated to generate localized products with several hundred copies of the ROI. These products are then probed using padlock probes, selective for mutant or wildtype variants of the ROI. Finally, reacted mutation-selective padlock probes are replicated via RCA. The localized products, having undergone two generations of RCA, are visualized via fluorescens-labeled detecting oligonucleotides for microscope examination. In paper IV, we have demonstrated that the method achieves prominent, accurate detection signals, revealing cells that harbor mutant transcripts in cell preparations and fresh frozen tissues.

Current methods for Covid-19 diagnosis

With the outbreak of coronavirus disease in 2019 (COVID-19), a variety of diagnostic techniques were available for SARS-CoV-2 infections. Developing rapid, accurate, and large-scale diagnostic tools became of great importance for proper epidemiology, patient management, and to prevent spread of the disease. The dominant diagnostic methods target theviral genome sequence or its protein products. Alternatively, patients' reactions to the virus can be investigated by measurement of antibody responses.

It is generally believed that nucleic acid amplification tests (NAATs) are the most sensitive tests available for detecting early viral infections, since viraemia is usually detected at a very early stage of the infection, and NAATs are generally both sensitive and specific. NAAT assays are therefore ideal for accurate diagnosis of clinical COVID-19 samples. Different NAAT assays involve reverse transcriptase PCR (RT-PCR), loop-mediated isothermal amplification-based assay (RT-LAMP), microarray, and high-throughput sequencing (Rai, Kumar, Deekshit, Karunasagar, & Karunasagar, 2021). As recommended by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), RT-PCR remains the gold standard method for detection of the SARS-CoV-2 virus, and one of the most commonly used method for population screening in different countries (WHO, 2020d; CDC, 2020i). A number of RT-PCR assays were developed, including ones targeting the nucleocapsid gene (N), spike gene (S), RNA dependent RNA polymerase gene (RdRp), ORF1b or ORF8 regions of the SARS-CoV-2 genome. The true positive rate of RT-PCR can be enhanced by targeting two or more gene sequences. It is important to note that the quality of viral RNA heavily influences the outcome of RT-PCR tests. Even though RT-PCR has been considered the gold standard for detection of SARS-CoV-2, it still has some limitations and issues that should be addressed in the future.

The term antigen refers to a molecule that activates the immune system to produce antibodies. Unlike PCR-based approaches, antigen tests use virus-specific antibodies to directly detect the virus or viral components (S glycoproteins, N proteins, etc). Similar to tests targeting viral RNA, antigen tests are only positive during the active stage of the viral infection, and the assays cannot reveal the status of recovery. Antigen detection by using lateral flow immunoassays can serve as rapid diagnostic tests but they are not as reliable as NAAT.

Detection of antibodies against SARS-CoV-2 in infected or vaccinated individuals is also an important diagnostic modality to check the immune status of people and provide complementary information. Immunological responses take longer to appear after infection than diagnostic RNA and antigen molecules. When the SARS-CoV-2 infection is in the acute stage and immune responses are not yet present, serological tests are of limited value for diagnosis

of SARS-CoV-2. Antibodies usually develop by 6 days after the onset of the symptoms, and an increase in antibody levels coincides with a decline in viral RNA levels (Lou et al., 2020). Serology tests identify IgM, IgG and IgA, which are produced in response to viral infection. No clear gold standard has been established for comparing the performance of different methods for detecting antibody responses. Also, there is no definitive evidence to relate the levels of detectable antibodies to immune protection (Safiabadi Tali et al., 2021).

In papers I and II of this thesis, we developed a sensitive detection method for SARS-CoV-2 antibodies in dried blood and saliva spots based on proximity ligation assays, using an approach different from that of commercial antibody tests. We also compare the sensitivity and specificity of this method with other methods for measurement of specific antibodies.

Present Investigation

Paper I. Detection of SARS-CoV-2 antibodies in serum and dried blood spot samples of vaccinated individuals using a sensitive homogeneous proximity extension assay.

Background

Since the outbreak of the COVID-19 pandemic in 2019, there has been a rapid development of assays to assess immunity to this severe acute respiratory syndrome, caused by SARS-CoV-2. Most assays involve a solid-phase, such as in lateral flow assays (LFA), enzyme linked immunosorbent assay (ELISA), and chemiluminescence immunoassay (CLIA) that are all form of ELISA. Among their advantages are the ability to detect both titers and isotypes of the antibodies. Frequently encountered problems in these ELISA are weak signals and limited dynamic ranges.

Homogenous proximity extension assays (PEA) are protein detection reactions that differ in several respects from more standard immune assays for protein detection (Assarsson et al., 2014). Like sandwich ELISAs, PEA depends on target recognition by pairs of affinity reagents, but no solid phases are needed for capture and no washes are required. Proteins are accurately quantified from levels below pg/ml and over wide dynamic ranges. Typically sample volumes of only 1 µl or less are used for multiplex assays of a hundred or more target proteins (Fredriksson et al., 2002). A related solution phase assay, agglutination-PCR (ADAP), has been developed for detection of total levels of SARS-CoV-2 antibodies (Karp, Cuda, et al., 2020). This assay consists of four reaction steps and a very good sensitivity of 98% and specificity of 99.55% was reported.

Collection of dried blood samples collected by finger pricks has proved to be a useful means of monitoring SARS-CoV-2 antibody levels, as samples can be collected at home and sent by post for analysis (Meyers et al., 2021; Morley et al., 2020). Previous analyses of dried samples have been limited by low sensitivity (Fontaine & Saez, 2021), the need for relatively large sample volumes (Omosule et al., 2023), or long and complex experimental procedures (Karp, Cuda, et al., 2020).

Aim of Study

A homogeneous PCR-based assay for sensitive and specific detection of antibodies in serum or dry blood samples (DBS) is presented, using a modified proximity extension assay (PEA) - AbPEA. The method was used to monitor individuals infected with or vaccinated against SARS-CoV-2. In the published report, the sensitivity, specificity and dynamic range of AbPEA were found to be very good, and applicable both to wet samples and DBS. The assay was benchmarked to other methods for measuring specific antibody responses.

Most important findings

AbPEA offers highly specific and sensitive solution-phase antibody detection without requirement for secondary antibodies and no elution step when using DBS samples in a simple procedure. Importantly, the assay lends itself for multiplex surveys of antibody responses against large sets of antigen. Anti-S1 and anti-NP antibodies were detected simultaneously in the same reactions. This antibody PEA (AbPEA) test uses only 1 µl of neat or up to 10,000-fold diluted serum, or a ø1.2 mm disc cut from a DBS. All 100 investigated sera and 21 DBS collected prior to the COVID-19 outbreak were negative, demonstrating a 100% specificity. Antibody positivity using this method was compared to results from more standard assays and the area under the curve, as evaluated by Receiver Operating Characteristic (ROC) analysis reached 0.998 (95%CI: 0.993-1) for samples taken from 11 days after symptoms onset. The kinetics of antibody responses were monitored after a first and second vaccination using serially DBS collected from 14 individuals, revealing the evolution of stronger immune responses over time.

Work Plan and Methods

In AbPEA, detection probes were prepared by conjugating the recombinant spike protein subunit 1 (S1), containing the receptor binding domain (RBD) of SARS-CoV-2, to each of a pair of specific oligonucleotides. The same was done for the nucleocapsid protein (NP). Upon incubation with serum or DBS samples, the bi-, tetra- or multivalency of the antibodies (IgG, IgA or IgM) brings pairs of viral proteins with their conjugated oligonucleotides in proximity, allowing the antibodies to be detected by this modified proximity extension assay.

Paper II. Monitoring SARS-CoV-2 IgA, IgM and IgG antibodies in blood and saliva samples using antibody proximity extension assays (AbPEA)

Background

Serological tests capable of monitoring immunity at the population level are of increasing importance as means to identify and monitor outbreaks of pandemics as well as to record the immune status of individuals. Previously, a sensitive homogenous antibody proximity extension assay (AbPEA) was developed for sensitive and specific detection of anti-SARS- CoV-2 antibodies from serum and dried blood spots (DBS). However, fingertip blood collection may still be considered somewhat invasive and uncomfortable, particularly for children. Self-collected saliva in dried form are therefore considered attractive alternative types of samples that may be collected easily and non-invasively without any discomfort. Saliva is a potential sample source for surveillance of immunity to SARS-CoV-2 because of the known strong correlation between antibody responses as measured in saliva and blood during acute infection and recovery. As there are currently few widespread procedures for dried saliva-based serology tests for SARS-CoV-2, new approaches are required to fill this gap. The GSP/DELFIA technique can detect saliva IgG in dried samples with 85.3% sensitivity and 65.7% specificity (Lahdentausta et al., 2022). The assay used 3.2 mm diameter punches from dried saliva samples on paper (DSS), corresponding to approximately 3 µl wet saliva.

Aim of Study

In this study, AbPEA was applied to dried saliva spots to determine the immune response in saliva. The high sensitivity, as well as reproducibility and stability of AbPEA for detection of antibodies in dried saliva samples were demonstrated using sets of samples. We investigated whether anti-S1 antibodies could be detected in dry saliva samples from all 42 individuals collected during Autumn in 2023, we also investigated how levels of antibodies in dry saliva correlated with those in DBS from the same individuals. Another objective of this study was to adapt AbPEA for measuring SARS-CoV-2 IgG, IgM and IgA subclass of antibodies from both dry blood and saliva samples.

Most important findings

Antibody could be detected directly from dry saliva sample discs from vaccinated individuals with no need for an elution step, as well as after 1000-fold dilution of eluted saliva. Dry saliva can be stored at room temperature for at least 6 days with no significant decrease of recorded antibody levels, which is beneficial for shipping by mail. Anti-S1 antibodies could be detected in dry

saliva samples from all 42 individuals sampled during the autumn of 2023. Total anti-S1 antibody levels measured from dry saliva samples correlate well with those from DBS. Antibody levels in dry saliva were on average 50-fold lower than levels in the corresponding DBS. We also show a modified AbPEA procedure, suitable for detecting the antibody subtypes IgG, IgA and IgM. Serially collected DBS from two individuals were used to monitor the kinetics of IgG, IgM, and IgA responses after a first and second vaccination, and we successfully detected IgA and IgG antibody responses using dry saliva samples collected in 2023.

Work Plan and Methods

AbPEA test was applied to the dry saliva samples in order to determine the immune response in saliva, and results were compared with those obtained from DBS. AbPEA was adapted to separately measure IgG, IgM and IgA antibody responses against SARS-CoV-2. A self- sampled series of DBS from two individuals from before and after a 1st and 2nd vaccinations were examined for IgA and IgG and dry saliva samples collected in 2023 were investigated as well.

Paper III. Surface protein profiles of extracellular vesicles reveal SARS-CoV-2 infection

Background

While diagnosis of COVID-19 rests on measurement of viral nucleic acids and proteins, measurement of other factors may also provide valuable information regarding the status of an infected individual and the progress of the infection. Extracellular vesicles (EVs) are nanometer-scale lipid bilayer-enclosed particles produced and released by almost all cells, and containing both proteins and nucleic acids, including microRNAs (miRNAs) and messenger RNAs (mRNAs). EVs have been proposed as promising biomarkers in a variety of diseases. For instances, miRNA isolated from small EVs have been proposed as biomarkers of chronic hepatitis B virus (HBV) infection (Sadovska, Eglitis, & Line, 2015). EVs have several useful properties as potential biomarkers, such as easy isolation from blood and good stability. However, the diagnostic value of EVs in COVID-19 has not yet been determined. Surface proteins of EVs play an essential role in recognizing and binding to target cells for ensuring fusion and uptake of EVs (Prada et al., 2016). The surface protein composition of EVs can also reveal their origin, and thereby identify tissues and processes affected by disease.

Aim of Study

The purpose of this study was thus to characterize how the surface protein composition of EVs might be affected by COVID-19. In order to obtain a comprehensive overviews of the protein composition of large numbers of individual EVs we utilized our previously developed PBA technology (Wu et al., 2019). The technique was applied to compare surface protein complements of EVs derived from blood of healthy individuals versus those from individuals with mild or severe COVID-19.

Most important findings

This is the first study to profile individual EVs from COVID-19 patients using a panel of antibodies against 181 surface proteins. We found that infected patients had significantly fewer EVs expressing any of 152 proteins compared to healthy individuals. Comparing EVs from patients in intensive care units (ICU) with healthy donors, significant differences in numbers of EVs were found for 177 proteins. 569 pairwise combinations of proteins significantly differed in frequency between normal individuals and COVID-19 patients, while the corresponding number for differences between normal individuals and patients at ICU, was 2179. Our study provided insight in surface protein profiles of EVs in SARS-CoV-2 infected patients, which may reflect host responses to the virus and help evaluate new treatments.

Work Plan and Methods

In this study, we applied a PBA panel, which allowed us to determine the presence of combinations of a total 181 membrane surface proteins on individual EVs. Here, some modifications were made to the standard PBA procedures for analysis of samples from individuals with COVID-19 or healthy controls. Comparing to standard PBA, we performed fixation of EVs in 3.7% (w/v) paraformaldehyde in PBS before antibody probing. We investigated whether surface protein patterns differed between EVs in samples from COVID-19 patients with mild or severe infection and from healthy individuals. We performed dimensional reduction of individual EVs from different sources based on their surface protein compositions using both T-distributed Stochastic Neighbor Embedding (t-SNE) and Uniform Manifold Approximation and Projection (UMAP).

Paper IV. Highly selective in situ detection of mutant transcripts by using superRCA and gap-fill probes

Background

Intratumor heterogeneity can be an important ground for therapeutic failure, drug resistance and poor survival in malignancies. It can therefore be of benefit for cancer patients if possible clonal heterogeneity within their tumors is characterized. Although sequencing total or partial tumor genomes provides information on thousands of somatic mutations and multiple subclones that are genetically related, these methods fail to preserve the spatial architecture and locate rare subclones. It is therefore essential to develop methods for in situ mutation detection that can enable genetic analysis of tumor heterogeneity with high sensitivity and specificity while preserving spatial context.

Aim of Study

The goal of the present study was to develop an efficient and highly selective method for localized detection of mutant transcripts in a manner that can be clearly resolved from any nonspecific background.

Most important findings

We demonstrated the application of gap-fill padlock probes and in situ superRCA to specifically reveal the presence of point mutant RNA molecules in preserved tumor cells and tissues. In this approach hundreds of localized copies of each detected target sequence are created by rolling-circle amplification (RCA) that are then genotyped by second round RCA. Distinction of sequence variants is therefore highly accurate, and any erroneous genotyping reactions by individual padlock probes remain undetectable. Once reacted the genotyping padlock probes are in turn replicated by RCA, providing prominent detection signals for each detected transcript. By using this method, we showed how the patterns of expressed mutated and wild-type alleles can be studied in situ in tumors with mutant KRAS transcripts. Compared with other methods, our analysis showed that this approach can improve the selectivity for sequence variants during in situ detection of mutant or wild-type transcripts, and the signals represented as superRCA reaction products are prominent and easily distinguished from any background.

Work Plan and Methods

In order to enable sensitive and highly selective RNA detection in situ, we illustrate an approach by using gap-fill probes and superRCA assays. First, cDNA synthesis is initiated from primers for reverse transcription of

sequences of interest, added to cells or tissue sections. The cDNA is then crosslinked to ensure proper localization. Next, RNA target sequences are removed by RNaseH-mediated degradation, and gap-fill padlock probes are added, targeting regions of interest in the reverse transcripts of the targeted mRNAs. Reacted probes form circular DNA strands that are locally amplified by RCA, generating localized products with several hundred copies of the ROI. The RCA products are then probed using padlock probes specific for either mutant or wildtype sequences present in the amplified copies of the target sequences. Finally, reacted genotyping padlock probes are replicated via a secondary RCA. Individual localized products from detected transcipts, having undergone two generations of RCA, are visualized via fluorescens-labeled detection oligonucleotides for examination by microscopy.

As a proof-of-concept we validated in situ superRCA assays for point mutations in codon 12 of the KRAS gene in cancer cell lines and in fresh-frozen tissues. The selectivity of the in situ superRCA assay for mutant versus wildtype transcripts was found to be superior to methods where genotyping padlock probes are used either to directly target transcripts or their cDNA copies before amplification by RCA.

Future perspective

Before starting PhD, I became extremely interested in in vitro diagnostic assays (IVDs). Currently, advanced molecular techniques are widely used for infectious disease diagnosis, cancer diagnostics and monitoring, personalized medicine and pharmacogenomics, identification of rare diseases, etc. In order to better understand molecular techniques for diagnosis and further improve several tools previously developed in our lab, I have been working with different research projects related to detection techniques for proteins, antibodies, and for mutant RNA.

Let's start with immunoassays, which were the subject of the first three papers. Proteins in general and specifically also antibodies are essential molecules for in vitro molecular diagnosis due to their critical roles and functions in signaling networks, cellular processes or immune defenses of relevance for disease processes. In our lab we have pioneered DNA-assisted immunoassays, in which DNA are conjugated to the desired pairs of antibody or antigen. Several DNA-assisted immunoassays are built on work from our lab, for example PLA and PEA, which apply DNA-assisted immunoassays with proximity-dependent principles. Although the aim of papers I and II did not include scaling the assay for parallel analysis of large sets of antibody specificities, the AbPEA mechanism is suitable for simultaneous analysis of reactivity to large sets of samples, and with a readout via sequencing this could be investigated for large sets of biobank samples in parallel. For detection of class-specific antibody responses in paper II, we have explored multiplexing AbPEA to investigate IgG, IgM and IgA responses in the same reaction in preliminary experiments. Broad screens for antibody reactivity could play a crucial role in preparing for and managing future pandemics, providing insights into the effectiveness of vaccination campaigns and helping to evaluate prevention strategies. In addition, regular antibody testing of the population can serve as an effective early warning system for the emergence of new pandemic. Antibody reactivity is also of diagnostic value in suspected autoimmunity, and multiplex AbPEA could fill a need for high-throughput techniques to assess the immune status of sampled individuals. We showed that AbPEA is suitable for measuring antibody reactivity in dried blood or dried saliva spots, rendering sampling low cost and high-throughput with preserved sensitivity, specificity and broad dynamic ranges.

Multiplexing is also an important feature of the proximity barcoding assay. The assay was designed to profile the surface protein composition of individual EVs. In this technique each detected protein on an EV is represented by a DNA barcode that can be sequenced to identify the protein and reveal the protein complex it originated from. The technique is suitable for very high-throughput studies of protein patterns on a large number of individual EVs (Wu et al., 2019). In paper III I investigated how the protein composition of microvesicles differed among blood samples collected from healthy individual or ones with either mild or severe COVID-19, demonstrating interesting differences. However, this technique still requires many optimizations, especially background problem. Additionally, sets of surface proteins of EVs identified by PBA as having a potential value as biomarkers, will need to be further validated in large numbers of sample. Here, more targeted methods are appropriate, and variants of proximity assays could be used. By identifying subsets of EVs characterized by particular constellations of surface markers it should become possible to investigate whether these small vesicles play a role in the transmission of molecular cargo during the course of Covid-19 or other diseases.

In paper IV, superRCA was for the first time applied for in situ analysis of cells carrying mutant transcripts. The method could prove of great value both for research and in clinical routine. By specifically detecting mutant RNA in tissues, pathologists may gain insights into the persistence or elimination of cells with specific mutations, providing valuable information about the effectiveness of the surgical intervention. In a research setting, this technique could be applied in multiplex with readout via in situ sequencing. This could allow for simultaneously identifying multiple mutations in a given tumor in order to characterize the evolution of subclones within the tumor and metastases. Additionally, by studying mutant transcripts and proteins simultaneously in situ, any differences in protein expression could be explored between normal and malignant cells in situ Work with this aim is already underway in the lab and can prove helpful information for spatial diagnostics.

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