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Molecular Tools for Biomarker Detection

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Abstract

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The advance of biological research promotes the emerging of new methods and solutions to answer the biological questions. This thesis describes several new molecular tools and their applications for the detection of genomic and proteomic information with extremely high sensitivity and specificity or simplify such detection procedures without compromising the performance.

In paper I, we described a general method namely super RCA, for highly specific counting of single DNA molecules. Individual products of a range of molecular detection reactions are magnified to Giga-Dalton levels that are easily detected for counting one by one, using methods such as low-magnification microscopy, flow cytometry, or using a mobile phone camera. The sRCA-flow cytometry readout presents extremely high counting precision and the assay's coefficient of variation can be as low as 0.5%. sRCA-flow cytometry readout can be applied to detect the tumor mutations down to 1/100,000 in the circulating tumor cell-free DNA.

In paper II, we applied the super RCA method into the *in situ* sequencing protocol to enhance the amplified mRNA detection tags for better signal-to-noise ratios. The sRCA products co-localize with primary RCA products generated from the gene specific padlock probes and remain as a single individual object in during the sequencing step. The enhanced sRCA products is 100% brighter than regular RCA products and the detection efficiency at least doubled with preserved specificity using sRCA compared to standard RCA.

In paper III, we described a highly specific and efficient molecular switch mechanism namely RCA reporter. The switch will initiate the rolling circle amplification only in the presence of correct target sequences. The RCA reporter mechanism can be applied to recognize single stranded DNA sequences, mRNA sequences and sequences embedded in the RCA products.

In paper IV, we established the solid phase Proximity Ligation Assay against the SOX10 protein using poly clonal antibodies. Using this assay, we found elevated SOX10 in serum at high frequency among vitiligo and melanoma patients. While the healthy donors below the threshold.

Keywords: Rolling circle amplification, padlock probe

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“Sharp tools make good work.”

-*Confucius*

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 **Chen, L.**, Björkesten, J., Wu, D., Mathot, L., Liebs, S., Haybaeck, J., Sjöblom, T., Landegren, U. A molecular approach for single molecule counting and rare mutation detection in blood plasma. Manuscript.
- II Wu, C., Mignardi, M., **Chen, L.**, Landegren, U., Nilsson, M. Profiling and genotyping individual mRNA molecules through *in situ* sequencing of super rolling circle amplification products. Manuscript.
- III Björkesten, J., **Chen, L.**, Landegren, U., Rolling Circle Amplification (RCA) Reporters – a new genetic tool for the detection of DNA, RNA and proteins. Manuscript.
- IV Blokzijl, A.*, **Chen, L.***, Gustafsdottir, S., Vuu, J., Ullenhag, G., Kämpe, O., Landegren, U., Kamali-Moghaddam, M., Hedstrand, H. (2016) Elevated levels of SOX10 in serum from vitiligo and melanoma patients, analyzed by proximity ligation assay. PloS One 11(4): e0154214 *Equal contribution.

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Abbreviations

ARMS-PCR	Amplification refractory mutation system-polymerase chain reaction
BEAMing	Beads, emulsion, amplification and magnetics digital polymerase chain reaction
CDKN2A	Cyclin dependent kinase inhibitor 2A
CDK4	Cyclin dependent kinase 4
CNV	copy number variation
C2CA	Circle to circle amplification
CPDs	Cyclobutane pyrimidine dimers
CVs	Coefficient of variation
DNA	Deoxyribonucleic acid
dPCR	Digital polymerase chain reaction
cfDNA	Cell-free deoxyribonucleic acid
ctDNA	Circulating tumor DNA
ExCircle	Extension and circular probe
FACS	Fluorescence-activated cell sorting
hRCA	Hyper-branched rolling circle amplification
HMG	High-mobility group
KRAS	Kirsten rat sarcoma viral oncogene homolog
MIP	Molecular inversion probes
MITF	Microphthalmia-associated transcription factor
MM	Malignant melanoma
NGS	Next generation sequencing
N-RAS	Neuroblastoma rat sarcoma viral oncogene homolog
PCR	Polymerase chain reaction
PEA	Proximity extension assay
PLA	Proximity ligation assay
PNA	Peptide nucleic acid
P53	Protein 53
PAX3	Paired Box 3
qRT-PCR	Quantitative real-time PCR
RCA	Rolling circle amplification
RNA	Ribonucleic acid
RHD	Rh blood group D antigen

SOX10	Sex determining region Y – Box 10
sRCA	Super rolling circle amplification
SRY	Sex determining region Y
Safe-SeqS	Safe-sequencing system
SNP	Single nucleotide polymorphism
spPLA	Solid-phase proximity ligation assay
S100B	S100 Calcium Binding Protein B
UMI	Unique molecular index
UID	Unique identifiers
UVB	Ultraviolet B

Introduction

The human genomic information is encoded by 3.08×10^9 bp of deoxyribonucleic acid (DNA) stored in the cell nucleus. But the actual functional activities across the whole body are mainly executed by the protein products. The connection between protein and DNA is linked through ribonucleic acid (RNA). As the central dogma of molecular biology states, there is constant information flow transmitted from the data storage (DNA) to the functional agents including RNA and proteins. Such information flow is dynamic and homeostatic within the cells.

The term biomarker refers to a measurable indicator of the biological or conditions. Both the static information stored in the DNA and the dynamic information of the RNA and proteins could serve as biomarkers to indicate physiological or pathogenic processes or pharmacologic responses. Depending on the questions that need to be addressed, direct acquisition of specimen from suitable organs is a straight forward approach to examine the biomarkers, complicated by the inaccessibility of most organs. Such specimen acquisition is usually destructive and irreversible. By contrast, studies of cells and molecules available in venous blood draw could serve as a convenient alternative to access tissue samples non-invasively.

The analysis of biomarkers requires the assistance of probes or approaches to convert the quantity or quality information of the biomarkers to machine readable signals. For DNA and RNA studies, PCR, probe based hybridization method and sequencing are the most popular tools to decode the sequence information. For protein studies, the protein recognition can be done by reading the sequences of amino acids using mass spectrometry or by employing affinity reagents to recognize the protein epitopes, which represent a combination of sequence and the structural information. Usually, dedicated enzymes are engaged to facilitate the recognition of the detecting target or enhancing the detection signal for readout.

Cell-free DNA (cfDNA)

Discovery of cfDNA

Back to the late 1940s, two French scientists, Mandel and Metais discovered the presence of cfDNA in blood plasma[1]. However, this observation did not attract attention before DNA was discovered as the 'hereditary substance' rather than protein by the famous Avery-MacLeod-McCarty experiment[2]. In 1966, for the first time, cfDNA was linked to disease[3]. Tan and his colleagues observed that the cfDNA level was high in the blood of systemic lupus erythematosus patients. One year later, Leon with colleagues demonstrated that the level of cfDNA was significantly higher in cancer patients' blood compared to normal control subjects.

In 1994, both Vasioukhin and Sorenson reported the presence of tumor-specific N-RAS mutations in cfDNA through their independent work[4, 5]. After that, more tumor specific DNA changes were discovered in cfDNA such as loss of heterozygosity[6, 7], gene amplifications[8, 9], presence of oncogenic viral DNA[10-12] and hyper-methylated promoter region of tumor suppressor genes[13, 14]. Moreover, in 1998, Denis Lo discovered male fetal DNA among cfDNA in maternal blood[15].

The presence of tumor specific and fetus-derived cfDNA enabled the development and application of cfDNA-based diagnostic approaches to characterize the malignancy in blood samples from tumor patients and the fetus in pregnant women. With a similar rationale, cfDNA was also applied to monitor the status of solid organ transplantations to search for signs of rejection.

Characteristics of cfDNA

The major source of cfDNA is cells that die from necrosis and apoptosis. These dead cells are routinely phagocytosed by macrophages and other scavenger cells, releasing small pieces of DNA into the blood stream and other body fluids. The double stranded cfDNA is highly fragmented with a major peak size at 166 bp. This suggests that the genomic DNA breaks in the units of nucleosomes, a basic element for DNA organization in the nucleus. And the other peaks in the cfDNA length distribution map are multiples of the nucleosome units (2 units =332 bp, 3units =498 bp)[16]. The amount of cfDNA in cancer patients' blood tends to be much higher than in the blood

of healthy controls and nonmalignant patients. However, the concentration of cfDNA in cancer patients varies considerably and for the majority of cancer patients, the value is lower than 100 ng/mL of blood[17].

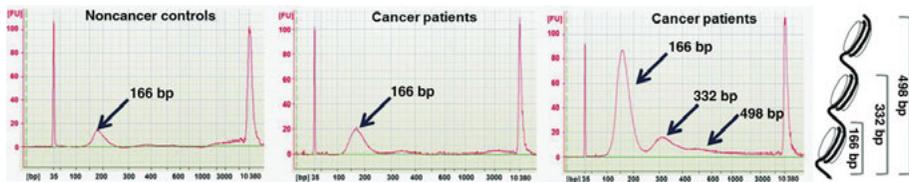


Figure 1. Size distribution of plasma DNA samples from healthy individuals and cancer patients[16]. The x-axis shows the size of DNA fragments and the y-axis represents the fluorescence intensity, which is proportional to DNA concentration. There is a major peak at 166 bp for all the samples, and the unusual trimodal distribution of DNA size (166, 332 and 498bp) indicates a very high tumor burden.

As expected, the mutant fraction of circulating tumor DNA (ctDNA) to total cfDNA also varies drastically. In a study by Diehl and colleagues of, a cohort of 33 colorectal cancer patients, the ctDNA fraction ranged from 0.01% to 1.7% of the total cfDNA[18]. While in another cfDNA study conducted by Collins and his colleagues, the AR gene mutation fraction of the metastatic castrate resistant prostate cancer patients varied between 0.1% to 23% with a median value of 1.5%[19].

The rapid turnover rate is another important characteristic of cfDNA. In 1999, Denis Lo and colleagues reported the half-life of male fetal cfDNA in woman post-partum as 16.3 minutes by detecting the SRY gene in cfDNA from mother's blood using real-time quantitative PCR assay[20]. In about 2 hours after birth, there is no detectable SRY signal from the mother's blood. But the mechanisms for cfDNA clearance in blood stream has not been studied in detail.

Methods for cfDNA analysis

Except for the highly fragmented property of cfDNA, there is no significant difference in character between genomic DNA isolated from cells and cell free DNA present in plasma. Methods work on genomic DNA from cells should also be applicable on cfDNA samples. cfDNA may include contributions from tumors in cancer patients[4, 5], from the fetus in pregnant women[15], and from transplanted organs in cfDNA from transplant recipients[21]. The minor contributions of cfDNA from these sources open up exciting new opportunities for molecular diagnostics. A simple blood draw can provide access to genomic DNA from tissues to be analyzed. And because of the ease of sampling, regular blood drawing provides possibility

to monitor the course of disease. However, the generally very low fraction of DNA from tissues of interest places demands on the methods to be applied to study them.

Quantitative Real-Time PCR (qRT-PCR)

In prenatal diagnostics, the qRT-PCR based cfDNA assay has been successfully applied in clinical laboratories for early detection of the blood group Rh genes[22] before the birth of a child.

Cancer genome alterations may consist of point mutations, insertions and deletions. In 1994, two groups reported the presence of tumor specific mutations in cfDNA using mutation-specific primers to facilitate amplification of N-RAS mutations in cfDNA. This is the first time that PCR method was employed in cfDNA studies[4, 5]. Researchers have been successfully detecting the presence of MYCN amplification by qRT-PCR in the late stage of patients[9]. However, this approach becomes inefficient for patients at stage I and II, probably due to the low abundance of ctDNA, as well as the limited sensitivity of the assay[23].

Cancer mutations, especially recurrent ‘hot spot’ mutations, are another technical challenge for qRT-PCR. Scientists have come up with several approaches to specifically amplify the mutant templates. For example, amplification refractory mutation system ARMS-PCR[24, 25], PNA clamping PCR[26], single base extension assay and ligation based mutation detection methods[27]. The central concept of these methods is either to create weakly binding primers such that only perfect matched primers can be extended, or to deplete out the wild-type strand during the PCR step, so only the mutant strands will give rise to PCR amplification products, or to utilize the substrate recognition fidelity of the ligase and polymerase to distinguish single base differences. However, the low abundance of ctDNA limits the performance of such methods.

Single base extension assay employs the fidelity of DNA polymerase to query the specific base information of the target strand. In this method, target sequences are pre-amplified with universal PCR primers without the discrimination of mutant and wild-type. The target strand of the amplified PCR products hybridized with a genotyping primer which sits one base upstream of the target. The dedicated DNA polymerase incorporate a fluorescent labeled dideoxy nucleotide to the genotyping primer and the fluorescent signal on the dideoxy nucleotide reflect the nucleotide information of the target base.

Ligation-based methods generate new DNA strands by joining 5’ and 3’ ends of oligonucleotides, subject to correct base pairing to a target strand. However, the accuracy of mutation detection is highly depended on the ligase fidelity. Thermophilic ligases can have fidelities of 99%, meaning that 1% of the DNA sequences would be incorrectly recognized by the ligation

reaction [28]. Thus, the ligation based method would not be able to detect low frequency ctDNA mutations due to false positives.

It is a similar case for methods like ARMS-PCR[24] and PNA clamping PCR[26]. The single nucleotide discrimination capacity of ARMS PCR depends on the selectivity of the PCR primers. In conventional PCR, the primer pairs are fully complementary to the templates. However, in ARMS-PCR, One of the ARMS PCR primer is placed right beside the mutation site with two adjacent upstream bases not complementary to the amplification templates. This primer is further destabilized by mismatches at the 3' end so that only one of the target sequence variants will yield PCR products.

In the PNA clamping PCR assay, apart from the regular primer sets for the PCR reactions, a peptide nucleic acid (PNA) based short oligonucleotides is included in the PCR system. The PNA/DNA duplexes are generally 1°C per base pair more stable than the corresponding DNA/DNA duplexes at the physiological ionic strength[26]. Accordingly, the PNA probe can block out the wild-type templates from PCR by creating a structure that prevents the DNA polymerase from replicating one sequence variant of the DNA templates. Meanwhile, PNA/DNA duplexes possess greater mismatch discrimination capability than regular DNA/DNA duplexes, so the alternate sequence variant is not affected by the PNA depletion and can be amplified effectively.

In general, these methods all have limited sequence discrimination capabilities that impose limits to the levels of rare mutant sequences that can be detected.

Digital PCR

To overcome the shortcomings of regular homogeneous PCR detection, Morley and Sykes published the first paper introducing the concept of digital PCR and Vogelstein and Kinzler fully developed the idea of digital PCR for mutation detections[29, 30], which extends the application of conventional bulk PCR. Using the digital PCR concept, each PCR reaction amplifies a single template rather than the large numbers of starting templates that are normally amplified in conventional homogenous PCR. The individual amplification and detection of the products of single templates minimizes the risks of errors introduced during the course of PCR. In digital PCR misidentification of single nucleotide variant templates can be as low as 10^{-5} . At a worst case, the polymerase may make a mistaken target recognition of one of the two template strands in the very first cycle. In this case one fourth of the PCR templates in subsequent cycles would carry the incorrect nucleotide information in a specific position of interest. This is an extreme case occurring only at very low frequencies. The majority of amplified products in the digital PCR reactions faithfully reflect the identity of the starting DNA template. The amplified PCR products are detected with fluorescent probes such

as molecular beacon that allows sequence specific detection using different fluorophores. By detecting fluorescent signals corresponding to mutant or wildtype sequences in each digital PCR reaction, the frequency of mutant sequences is easily counted.

The next question is how to ensure the presence of single copy template molecules in the digital PCR reactions. Vogelstein and Kinzler dilute the templates extensively in the PCR mixture so that on average 0.5 template molecule (haploid genome equivalent) would be expected per reaction in the format of 96 well PCR plates. This means that 50% of the wells would contain one copy of the template. However, in this kind of setup, on average 48 templates are the maximum input per 96 well microtiter plate, limiting the throughput of the assay. Different solutions have been presented to generate more digital PCR reactions per run by downsizing the reaction volumes. Several groups successfully performed digital PCR reactions in microfluidic chips with tens of thousands of micro compartments[31-33]. Other formats of compartmentalization have also been demonstrated such as microarrays[34], cavities in spinning microfluidic discs[35] and water-in-oil emulsions[36]. Some years later, the company RainDance pushed the size limit of water-in-oil emulsions from nano-liter to pico-liters, resulting in up to 10 million emulsions per reaction[37].

Vogelstein and his colleagues developed a variant of digital PCR called BEAMing based on four components: beads, emulsion, amplification and magnetics[38]. Unlike conventional digital PCR, BEAMing assays include a step of pre-amplification with a high fidelity DNA polymerase to expand the copy numbers of each template to avoid the loss of single target in the experimental procedures and to increase the counting precisions when mutant numbers are low. Each PCR template together with magnetic beads, and PCR reagents are encapsulated into oil emulsions. With another round of bead based colony PCR, magnetic beads are collected and purified with a magnet after breaking the emulsion. Next, DNA captured on magnetic beads is denatured, and a single base extension assay is performed on the DNA clusters yielding fluorescent signals reflecting mutant or wild-type sequences. The fluorescent signals on the magnetic beads are detected by flow cytometry.

The pre-amplification step in the BEAMing assay carries a certain risk of introducing mutation by the DNA polymerase. For a specific base, a polymerase error rate of 1.95×10^{-5} after 30 cycles for a high-fidelity polymerase is much lower than the mutation frequency possible to detect in the limited amount of DNA available in a plasma sample (1/10,000 for 33 ng cfDNA input). The assay performance therefore is limited by the number of DNA molecules present in the sample rather than by the polymerase error rate. The BEAMing technology has been reported in several publications for mutation detection of ctDNA samples in various cancer types.

Sequencing based methods

The targeted approach limits the possibility to assess a comprehensive mutational landscape of tumors creating the need for a more generalizable technique suitable to detect any sequence variant in the template.

The Sanger DNA sequencing method has been the gold standard to analyze DNA sequence information, such as mutations in germ-line and in expression constructs. Due to the technical limitation of Sanger sequencing, mutant allele can only be detected when the fraction is greater than approximately 20%[29]. However, the mutation fraction in ctDNA is usually lower than 1%, and sometimes it can be as low as one single molecule in the entire sample.

The advent of next generation sequencing expands the tool box for genomic studies. Next generation sequencing represents a high-throughput approach through massively parallel processing. In this method DNA templates are randomly fragmented and single molecules are amplified in colonies by methods such as beads based emulsion PCR[38], or rolling circle nanoballs[39], or by bridge PCR[39] where both the forward primer and reverse primers are covalently attached at high-density to slides, or by so called isothermal template walking in the SOLiD platform where one primer was anchored on the surface and the library is amplified through isothermal PCR with strand displacement DNA polymerases. After clonal amplification, the sequence information is decoded by sequence-specific addition of labeled or unlabeled nucleotides to a growing strand or by target-specific ligation reactions. The NGS mutation detection performance is greatly improved compared to the traditional Sanger sequencing approach. However, in hunting for ctDNA mutations, 0.5% sensitivity still leaves a large space for improvement.

NGS has been a promising alternative for quantifying ctDNA, but the higher error rate relative to the accuracy of digital PCR and the low coverage in depth hinder its application. Cancer related mutations are more likely present in the coding regions, focusing the sequencing power on the informative regions has been a major effort. Region-specific PCR and hybridization-based capture of target region allows greater sequencing depths so that the relative amount of mutant and wild-type DNA molecules at each locus may be counted accurately.

Several approaches have been applied to further increase the fidelity of next generation sequencing, and accordingly the ability to detect low mutation frequencies, such as by employing a high-fidelity PCR polymerase and decreasing the number at PCR cycles. The most promising strategy to further suppress the sequencing error involves unique molecular index (UMI) also known as barcoding or unique identifiers (UID). The UMI concept is similar to the digital PCR concept, through the isolation of each input template, the amplified products are grouped and analyzed as a family. In digital PCR, this

concept is fulfilled by physical isolation and grouping of the input molecules, such as water-oil emulsion and micro fluidic chips. In the sequencing approach, the isolation is done by labeling individual molecules with UMI consisting of DNA molecules with random bases through PCR or ligation. For example, in the ‘Safe-SeqS’ protocol, the target regions are enriched by PCR[40]. The amplicon primers are designed with two functional segments, the 3’ segment is amplicon specific and the 5’ part is universal across all amplicons. The UMI sequence is only present in the forward primer between the universal and the target specific parts. When the target region is pre-amplified, in the first 2 cycles, the forward primer carrying the UMI extends on the template and in a second cycle the reverse primer extends by copying the UMI-tagged extension products. The first-round primers are removed using a single-strand specific DNA-exonuclease and then universal primers are applied to continue the amplification. The sequence reads amplified from the same target strand will carry the same tags in the 5’ end and the actual sequence is determined by the consensus sequence within each UMI family for a given target sequence. It is important that the number of distinct UMIs greatly exceeds the number of original templates to minimize the probability that two distinct templates would acquire the same UMI. And it is for the same purpose that the template concentrations should also be kept relatively low. In the analysis of ctDNA sequencing data, researchers have found that particular sequencing artifacts are not suppressed by UMI-assisted sequencing, such as guanine (G) to thymine (T) changes are more prevalent than cytosine (C) to adenine (A) changes. This must be dealt with by filtering through bioinformatics. The ‘Safe-SeqS’ protocol has successfully pushed the mutation detection limit to 0.02% and other methods together with dedicated bioinformatics algorithms have further improved the sensitivity to reach still lower mutation frequencies.

Padlock Probes and Rolling Circle Amplification

Padlock Probes

A padlock probe is a short DNA oligonucleotide with segments at the 3' and 5' ends that are complementary to a target region[41]. Upon hybridization, the two ends of the probe oriented in juxtaposition on the target template, leaving a nick site in the double-stranded structure. The nick site is sealed by a DNA ligase, and thereby the padlock probe is wound around and locked on the target strand. The DNA ligase activity is sensitive to base pair mismatches around the nick site, empowering the single base discrimination capacity of the padlock probes[42]. The central part of the padlock probe is not target complementary and can harbor specific sequences serving different purposes, such as a detection probe hybridization site, sites for amplification primer hybridization, and capture probe binding site [43-47]. Padlock probes have been used in many applications, for example for copy number variation analysis (CNV)[48], single nucleotide polymorphism (SNP) analysis[49-51], gene expression profiling[52-54], alternative splicing analysis[55] and pathogen detection[56, 57].

Molecular inversion probes (MIP) are variant of the padlock probe concept[58]. When the two ends of the MIP hybridized to its target, a gap is formed instead of a conventional nick. The length of the gap can vary from a single nucleotide to hundreds of nucleotides for different probes. A DNA polymerase lacking strand displacement activity fills the gap by priming from the 3' end of the MIP. The gap-fill specificity is provided by the fidelity of the DNA polymerase, and target selectivity can be further augmented by the subsequent ligation step[59]. Molecular inversion probes have been applied for highly multiplex genotyping[60] and high multiplex gene copy number measurement[61-63]. In one study, MIP were utilized to retrieve 10,000 human exon sequences with the gap-fill size ranging from 60 bp to 191 bp[64].

Selector probes are another variant of the padlock probe principle. After specific restriction enzyme digestion and DNA denaturation, selector probes serve to template enzymatic circularization of the restriction digested target DNA strands, turning the target sequences rather than the probes into closed DNA loops upon probe-target interaction[65, 66]. The circularized DNA

strands can then act as templates for RCA (see below) to yield single-stranded DNA products containing thousands of copies of the specific sequences for targeted genome enrichment. The selector probes lend themselves for enrichment of large numbers of sequences of interest for e.g. DNA sequence analysis.

Rolling Circle Amplification

Rolling circle amplification (RCA) is an isothermal amplification method, which generates strands containing thousands of repeats complementary to the DNA circle that serve as template for replication [67]. The single-strand clustered amplification products can be labeled with fluorophores or chromogenic functional groups through oligonucleotides hybridization. In combination with the molecular tools that generate circular reaction products like *in situ* PLA[68], padlock probes[69], selector probes[65], PLAYR[70] and others, RCA can serve to magnify detection events locally into highly visible signals.

Linear amplification of RCA imposes some limitations on the instruments that can be used for counting the molecules that form, as the signal intensity is limited by the numbers of repeats generated. Microscope imaging together with the image processing algorithms have been the major approach for quantification of RCA products. It is possible to enhance the fluorescent signal intensity by incorporating fluorophore linked nucleotides during the polymerization step but this does not permit multiplex analyses.

Circle to Circle Amplification (C2CA)

Dahl and colleagues developed a method to boost the number of RCA monomer products called C2CA[71]. In this approach, RCA products are first generated from circular reporting molecules that have been produced in various detection reactions. Then the RCA products are digested into monomers by restriction enzyme in the presence of short oligonucleotides complementary to the RCA concatemer. After heat denaturation, the monomers that form hybridize to new copies of the oligonucleotide, now serving as a ligation template. After the molecules have turned into DNA circles (complementary to the starting circles) the same oligonucleotide is ready to serve as primers for RCA. The process can be repeated, and can yield either single stranded concatemers or monomers of either polarity, as desired. For starting DNA circles of 100 nts each generation of C2CA yields a thousand-fold amplification in an hour of replication, and the generations may be repeated.

Hyper-branched Rolling Circle Amplification (hRCA)

In the hyper-branched rolling circle amplification, an extra reverse primer complementary to the RCA products is also present during the RCA step, besides the circular reporting molecule and the standard forward primer[72]. Hyper-branch RCA is initiated by hybridization and extension of the reverse primer on the RCA products, displacing any downstream strands in the direction of polymerization [73]. The newly displaced strands serve as templates for the extension of the forward primers, displacing strands in the polymerization direction. hRCA requires a pre-cleaning of the linear reporting molecule to avoid non-ligation induced hyper branch amplification. The products that form represent a combination of single and double stranded mono- and polymers of the starting DNA circle and its complement.

Primer Generation Rolling Circle Amplification (PG-RCA)

In the primer generation rolling circle amplification[74], pre-formed mature single strand DNA circles are embedded with target sensor sequences and enzymatic nicking sequences. Once the circle hybridized to the target sequence, Phi29 DNA polymerase synthesizes the long concatemer complementary to the circular template. Then multiple circular probes hybridized to the single strand concatemer and nicking enzyme recognition sequences are activated by double strand hybridization. Nicking enzyme nicks on the RCA product strand and yields multiple complexes of primer and circular probe subject to RCA and initiate the next round primer generation rolling circle amplification.

Enhanced Rolling Circle Amplification

There is a need for new amplification method that be used to could amplify the circular detection products to single entities containing millions or even more repeats for ease of detection, by taking advantage the clustered nature of RCA products. We conceived a RCA cascade where further generations of RCA products may be grown from a first RCA product, simplifying detection and augmenting detection specificity. Unlike other RCA-based methods, the clustered RCA products remain in a single-stranded state so the sequence information can be read out by oligonucleotide hybridization. In the rolling circle replication, the following components are required: a circular DNA template, a primer, a polymerase and suitable buffer components as in a regular RCA. To produce further generations of RCA products, the reactions also requires padlock probes that can recognize a first-generation RCA products and an oligonucleotide to prime their replication.

Primer dependent RCA enhancement (RCA reporters)

The general concept of the primer dependent RCA strategy is the availability of the primers for the subsequent RCA is dependent on the synthesis of first round RCA, while the circular template is provided externally. In the conventional RCA, there is no extra free 3' end generated apart from the only 3' end elongated from the primer if the RCA is intact as a single concatemer. The primers for the subsequent RCA cannot be generated directly from the RCA rather than provided conditionally during the process of first rolling circle amplification RCA if the RCA products stay intact. This primer dependent RCA approach is named as 'RCA reporters', and the RCA reporter consist of an intact circle plus a blocked primer molecule. The RCA reporter molecule is incapable to initiate the RCA when it solely incubates with the rolling circle solution with all necessary ingredients. When the RCA reporter is hybridized to the first-generation RCA products, the blocking group is removed from the primer of the RCA reporter. The subsequent RCA is initialized and the RCA products is still anchoring to the first-generation RCA products. We have designed and experimentally validated several strategies for the target dependent blockade removal (Figure 2), for example, nick design, hybridization design, circle-in-circle design and double circle design.

Nick design

Nick design is composed by a linear primer hybridized with a complete circle. In the absence of target template, the 3' end of the primer is incapable to initiate the RCA as the blocking region composed with phosphorothioate modified bases (red region) is not complementary to circle and resist to the exonuclease activity of the Phi29 DNA polymerase. When the anchoring sequences (blue region) and the RE sequences (orange region) hybridized to the target sequence, a nicking enzyme recognize the embedded sequences in the RE sequences and nick on the primer strand. The residual RE sequences on the primer fall off the target sequence and exposed to the exonuclease activity of Phi29 DNA polymerase. When the Phi29 DNA polymerase reach to the double strand formed by the primer and the circular template, Phi29 DNA polymerase switch to the polymerase activity and proceed to RCA activity.

Hybridization design

Similar to the Nick design, the *hybridization design* is also composed with a single strand primer and a complete circle. But in this concept, the protection of the 3' end of the primer is by the hybridization of lock sequences. In the presence of lock sequences and free dNTPs, the 3' end of the primer is protected against the exonuclease activity of Phi29 DNA polymerase. When the anchoring sequences (blue region) on the probe binds to the target sequences, part of the lock sequences (orange region) bind to the target sequences, this initiate the invading of the lock sequences to further bind to the target sequences as the lock sequences shares longer complementary to the target sequence than to the primer. This result in the complete exposure of the 3' end of the primer, thus the Phi29 DNA polymerase degrade the single strand part of the primer and proceed to RCA once it reaches to the double strand junction formed by the circular template and the primer.

Circle in Circle design

The *circle in circle design* is basically an improvement of the nick design. The 3' end protection is further improved by connecting the 5' end and 3' end of the primer to form a complete circle. In this case, there is no free 3' end in the system which is incapable to initiate the RCA in the single stranded template and stay intact in solution. In the presence of the target sequence, the nicking enzyme nicks on the RE sequences (orange region) on the hybridized double strand and release the 3' free priming end after nicking. The residual RE sequences on the primer fall off the target sequence and exposed to the exonuclease activity of Phi29 DNA polymerase. When the Phi29 DNA polymerase reaches to the double strand formed by the primer

and the circular template, Phi29 DNA polymerase switch to the polymerase activity and proceed to RCA.

Double Circle design

The *Double circle design* is further developed via constructing a fully hybridized circular template by providing the linear template with full sequence complement to the single strand circle. The 3' end and 5' end of the linear template share complement sequences and hybridized. The 3' end of the linear template is composed with phosphorothioate modified bases (red region) and is not complementary to the anchoring sequences (blue region) on the 5' end. In the presence of target sequences, the hybridization of the anchoring sequences promotes the dissociation and re-hybridization of the locking sequences (orange region) and priming sequences (red region) to the target sequences. The exposed priming sequences (red region) on the circular template is recognized by the 3' end of the linear template and initiate the RCA.

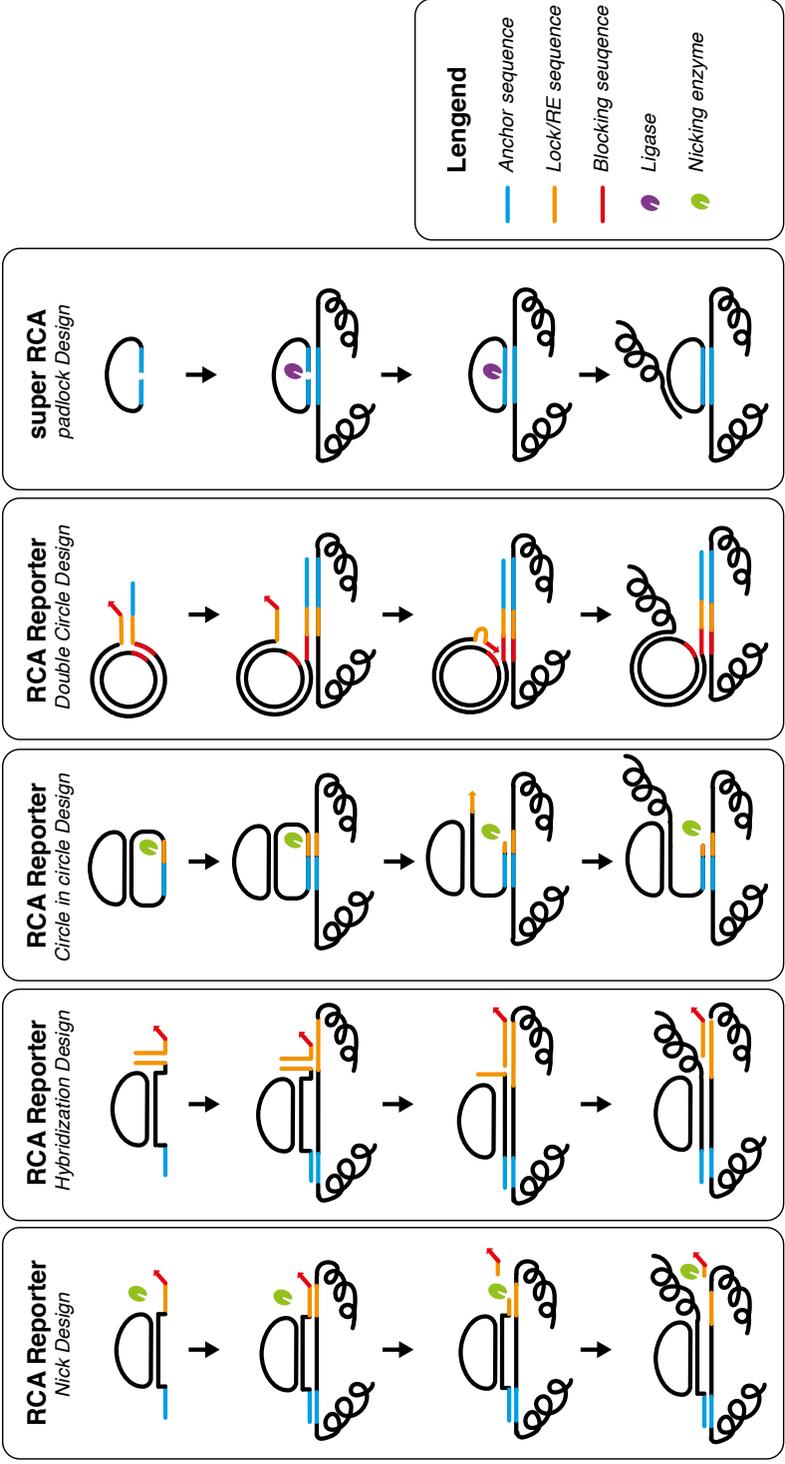


Figure 2. Illustration of RCA reporter and sRCA designs that have been experimentally tested.

Circle dependent RCA enhancement (super RCA)

In the circle dependent concept, the formation of intact circular template is dependent on the generation of first-generation RCA products. The linear secondary padlock probe hybridizes to the first-generation RCA products and ligated into circular template by DNA ligases. In the presence of externally provided primers, the ligated padlock probe initiates the RCA and yield RCA products winding around the first-generation RCA products. This approach is named as super RCA (sRCA). Permitted by the single nucleotide discrimination sensitivity of the padlock probe, sRCA is capable of genotyping hundreds of repeats of the first-generation RCA products, allowing any mismatched ligation products to be ignored. This yields for every starting DNA circle an sRCA product with great numbers of repeats that identify a variety of target sequences differing by as little as single nucleotide positions. In this manner, each starting DNA circle yields a million or so repeated DNA sequences in a cluster with micrometer dimensions, and a molecular weight in the tens of billion Daltons. Thereby, the sRCA products may be conveniently detected and enumerated with high precision using widely available laboratory equipment.

Both the sRCA design and the RCA reporter design can yield clustered RCA products represent every starting DNA circle. In the sRCA design, the RCA products templated padlock probe ligation need a DNA polymerase free condition to avoid unwanted extension primed by the un-ligated secondary padlock probe. Therefore, the single tube sRCA protocol is divided into three steps to separate the rolling and ligation. The RCA reporter protocol is both single tube and single step protocol as no extra ligation is required. The sRCA design is capable of detecting single nucleotide difference as a result of the secondary padlock probe ligation. In the RCA reporter design, target is recognized via hybridization, so it's very difficult to reach single nucleotide sensitivity.

These two designs can be applied in different themes, simple and fast signal amplification are the advantages of RCA reporter design, like signal enhancement in the conventional padlock probe assay and *in situ* PLA assay. sRCA design can be applied to the scenarios where amplification together with single nucleotide discrimination capability are needed for example rare mutation detection and high precision digital counting. The three-step sRCA protocol only requires the addition of reagents for the next step, so it can be automated easily by pipetting robot.

Table 1. *Methods for ctDNA mutation analysis.*

Platform	qRT-PCR		Digital Counting				Sequencing			
	Taqman probe /ARMS	PNA clamping	Bio-Rad	Life Technologies	Raindance	BEAMing	sRCA	Sanger sequencing	Next gen sequencing (NGS)	UMI-NGS
Sensitivity	++		+++	+++	++++	++++	+++++	+	+++	++++
Multiplexing	+		+	+	+	+++	+++	++	+++	+++
Compartmentalization	Homogenous		Emulsion	Microfluidic chips	Emulsion	Beads based emulsion	/	Homogenous	Homogenous	Unique Molecular Index
Numbers of Partitions	/		20,000	20,000	~ 10 million	500,000	~ 20 million	/	/	~30 million
Throughput	+		++	++	+++	+++	+++	++	++++	++++
Cost	+		++	++	+++	+++	+	+	++++	+++++

Melanoma and solid phase PLA

Melanoma

Malignant melanoma is a skin cancer that originates from pigment-containing cells known as melanocytes. Apart from the skin, melanoma can also be found in other organs of the body, like mouth, intestines and eyes. Since the melanoma cells produce melanin, they typically appear as brown and black neoplasms. Melanomas that do not produce melanin can be pink, tan or even white[75].

Ultraviolet light exposure to the low levels of skin is the primary cause of melanoma[76]. Ultraviolet UVB light with the wavelength between 280 -315 nm can cause a type of DNA damage involving cyclobutane pyrimidine dimers (CPDs) that can result in skin cancer[77]. After exposure to UVB light, two adjacent pyrimidines on the same DNA strand can be cross linked through C=C double bonds to create thymine-thymine, cytosine-cytosine, and cytosine-thymine dimers. T-T damages can be correctly replicated, but cytosine residues in dimers are prone to be deaminated, introducing a C to T transition[78].

Another cause for malignant melanoma is the presence of inherited mutations that greatly increase melanoma susceptibility. A typical example is mutations present in the CDKN2A gene[79]. One mutation in CDKN2A results in a reading frame change and leads to the destabilization of P53, a transcription factor involved in apoptosis[80] and another mutation in CDKN2A yields a nonfunctional inhibitor of CDK4, a cyclin-dependent kinase that promotes cell division. These inherited mutations diminish the ability to repair genetic lesions [81].

SOX10 is a transcription factor belonging to the E subgroup within the SOX family. It is highly expressed in the neural crest and later in the developing peripheral and central nervous systems[82]. Cells derived from the neural crest are multipotent giving rise to neurons, glia cells of the peripheral nerve systems, melanocytes of the skin, and cartilage and bone of the face[83]. SOX10 together with PAX3 regulate the promoter of the microphthalmia-associated transcription factor (MITF) gene and the MITF gene plays a central role in the development of melanocytes[84, 85]. Considering the expression patterns of SOX10 and its central role in the differentiation of melanocytes, we hypothesized that the level of SOX10 protein in serum might correlate with the disease status in melanoma.

Solid phase PLA(spPLA)

The concept of proximity based protein detection assay was first demonstrated by Fredriksson and colleagues in 2002[86]. Two DNA aptamers extended with distinct oligonucleotide sequences were used to bind the target protein PDGF. This brought the modified aptamers in proximity, permitting the oligonucleotide extensions to be joined by ligation. The newly formed DNA strand could be quantified by real-time PCR, reflecting the identity and amount of the target protein in a sample. The requirement for the presence of two binders to generate the reporter molecule greatly improves the specificity of immunoassay over assays dependent on binding by single reagents. The so-called proximity ligation assay was further developed by using conventional antibodies with conjugated DNA strands, expanding the scope for applications of the assay[87].

In the solution phase proximity ligation assay, the efficient detection of target relies on the dilution of the reaction solution after a first incubation of samples with the DNA-modified affinity reagents. This serves to reduce the probability of ligation of oligonucleotides on detection reagents that have failed to bind in proximity. The performance of the assay can be impaired by components in the sample inhibiting the ligase activity. A solid phase with pre-immobilized capture antibodies can be used to capture the target molecule from the samples, followed by the addition of a pair of oligonucleotide-conjugated antibodies[88]. Thereby, the incubated samples and excessive probes are all washed away before ligation. By using pools of detection probes, solid phase PLA has been used to detect up to 48 different analytes in a single sample aliquot, with readout via qRT-PCR[89] or next generation sequencing [90].

Present Investigations

Paper I: A molecular approach for single molecule counting and rare mutation detection in blood plasma.

Introduction

The ability to observe, evaluate and count even extremely rare macromolecules directly in biological samples helps to answer questions in biological research. The detection of mutant DNA or RNA molecules in plasma or distributed in tissues in tumor patients, and highly precise, digital enumeration of proteins and other molecules of interest in clinical specimens benefit from such technological advances. However, this need is poorly met due to limited availability of suitable tools[29, 38, 91, 92].

RCA is a well-known isothermal mechanism for nucleic acid replication, yielding for each circular template a single-stranded concatemer, composed of complements of the circular DNA strand. Several assays exist where DNA circles result from specific detection of a variety of biomolecules for convenient detection via RCA. For example, padlock probes are linear DNA strands that are converted to DNA circles in ligase-mediated DNA detection reactions[41, 59, 93].

The linear amplification of RCA limits its application in biomolecule counting as the signal intensity is limited by the available repeats. Microscope imaging together with the imaging processing algorithms have been the primary approach for the quantification of RCA products.

Aims of Study

The aim of this project was to develop a method to locally amplify individual detection reaction products with extremely high sequence specificity to form molecular clusters that can be recorded using standard lab equipment. The clusters would consist in localized products of two or even three generations of RCA if required.

Summary of Findings

We have proven that the sRCA concept is feasible and two or even three generations of RCA can be overlaid on single products of detection reactions to produce prominent localized amplification products. When labeled with chromogenic functional groups, the sRCA approach can convert single biomolecules into colored spots that can easily be recorded using a standard smartphone camera. The sRCA approach can also be combined with the *in situ* PLA assay to enhance the signals without any additional background.

By virtue of the specificity of a secondary padlock probe ligation using the fidelity of a DNA ligase, the sRCA approach can discriminate single nucleotide difference between the targets and be applied for detection of very rare tumor-derived point mutant DNA molecules in sample of cfDNA. As the secondary generation of RCA products remain attached to the first generation of RCA product, each sRCA product represents one starting DNA circle. When sRCA products are labeled with fluorescent oligonucleotides, the sRCA products can be recognized and digitally counted by flow cytometry. This sRCA-flow cytometry readout presents extremely high counting precision and the assay's coefficient of variation can be as low as 0.5%. sRCA-flow cytometry readout can be applied to detect the tumor mutations at frequencies as low as 1/100,000 in cfDNA. sRCA protocol requires three consecutive reagent additions in a single tube format, and the procedure lends it to be fully automated with pipetting robot.

Paper II: Profiling and genotyping individual mRNA molecules through *in situ* sequencing of super RCA products.

Introduction

Complex organ tissue consists of mixed population of cell types presenting distinct gene expression profiles[94, 95]. Outlining such gene expression pattern across the tissue helps to understand the cellular components of the tissue as well as tissue-specific gene expression patterns. Next generation sequencing (NGS) based RNA sequencing provides a comprehensive view of transcripts and their splice variants[96]. However, homogeneous sequencing fails to preserve spatial information of the transcripts, making it impossible to reconstruct the heterogeneous distribution of cells across the tissues. Several single cell isolation methods such as by FACS sorting or laser-capture microdissection have been applied to sequence transcripts of individual cells[97-99]. However, these approaches are limited by sampling bias and loss of histological context.

We have recently developed a technology for localized sequence library preparation with RCA as an approach for *in situ* sequencing[100, 101]. This method involves generation of clonally amplified and specially confined substrates for next-generation sequencing within the preserved context of cells and tissues. Our approach combines padlock probing, RCA, and sequencing-by-ligation chemistry to resolve expression profiles of sets of genes and mutations in tissues without loss of histological context[96, 102]. Like other fluorescence-based assays, the assays can be compromised by high levels of background fluorescence[103]. There is a tendency for amplified sequencing templates with weak signals to fall below the detection threshold due to the increased fluorescent background during the repeated sequencing cycles. As the base calling of *in situ* sequencing relies on the detection of fluorescent signals, this greatly compromises *in situ* sequencing quality and decreases library coverage.

Aim of Study

The aim of this study is to apply the sRCA signal amplification concept into the *in situ* sequencing method to achieve high signal-to-noise ratios.

Summary of Findings

In *in situ* sequencing, transcripts are decoded by reading the tags present in the amplified RCA products. The adaptation of sRCA signal amplification with *in situ* sequencing requires the encoding of the *in situ* sequencing tags

into the sRCA products. Several strategies including padlock probe with degenerated ends, padlock probe gap-filled with oligonucleotide insertions and padlock probe gap-filled by polymerization have been validated to compare the efficiency. Padlock probes gap-filled by polymerization presents the best efficiency and were selected for further optimization.

The sRCA products co-localize with primary RCA products, generated from the gene specific padlock probes and remain as a single individual object during the sequencing step. The enhanced sRCA products are 100% brighter than regular RCA products and the detection efficiency at least doubled with preserved specificity using sRCA compared to standard RCA. The sRCA enhanced *in situ* sequencing protocol has been successfully applied on cell line and tissue samples with improved S/N ratio.

Paper III: Rolling Circle Amplification (RCA) Reporters – a new generic tool for the detection of DNA, RNA and proteins

Introduction

RCA is a well-known isothermal mechanism for nucleic acid replication, yielding for each circular template a single-stranded concatemer, composed of complements of the circular DNA strand. However, the linear amplification characteristics of rolling circle amplification limits the total yield of the amplification output to thousand-fold and restrict the widely application of the method[41, 59].

Super RCA (sRCA) can yield million-fold or greater signal amplification of circularized detection probes in a localized fashion. However, the need for a secondary padlock probe ligation, necessitates performing a first and second-generation RCA consecutively. Accordingly, the procedure requires several steps to achieve the amplified signals.

Aim of Study

The aim of this project was to design a highly specific and efficient molecular switch mechanism referred to as RCA Reporters. The switch can initiate an RCA only in the presence of the correct target sequences. We will combine the so-called RCA reporter molecular switch with the proximity extension assay to develop the PEA products into flow cytometry readable RCA products as a digital counting alternative for the PEA assay.

Summary of Findings

We have come up several designs of the oligo system under the RCA reporter concept. The design has been finalized after the validation and further verification in different conditions. The RCA reporter molecules are pre-manufactured by assembling a padlock probe and a ligation template together in the presence of a DNA ligase. The target recognition sequence is pre-embedded in the ligation template near the 3' end. The sealed padlock probe and the RCA primer are protected in double strand structure by hybridization of protection sequences to prevent unwanted initiation of RCA. In the absence of the target, the RCA reporter remain silent. Once the target sequence is recognized by the target binding sequence of the RCA reporter, the protection sequence falls off and expose the primer. The primer competes off the protection sequence on the padlock probe and initiate the RCA in the presence of DNA polymerase. The RCA reporter originated RCA products remain linked to the target sequence and can

form RCA product clusters if initiation sequence is embedded in the RCA products. Such RCA clusters stay intact in solution and can be recognized as an intact object by flow cytometry.

Paper IV: Elevated levels of SOX10 in serum from vitiligo and melanoma patients, analyzed by proximity ligation assays.

Introduction

Malignant melanoma (MM) is a cancer that originates from pigment-containing cells known as melanocytes. The diagnosis of malignant melanoma starts with visual inspection and is then followed by a skin biopsy. The serum marker S100B protein is the most commonly used marker for monitoring tumor responses to treatments in MM patients in later stages and in recurrent disease[104]. But serum markers for clinical detection of early stage malignant melanoma are still missing. S100B lacks detection specificity and levels of the protein correlate poorly with prognosis.

Vitiligo is a pigmentation disorder...

SOX10 is an important transcription factor for normal development and function in melanocytes[105] and nerve cells. It has been reported to be essential for neural crest cell fate decisions[106], and it plays an important role in the development of giant congenital nevi and melanomas[107]. SOX10 is a member of the HMG (high-mobility group) box superfamily, and it belongs to the E subgroup of the SOX family. Mutations in the SOX10 gene are associated with the Waardenburg-Shah syndrome and Hirschprung's disease[108-110]. Expression of SOX10 mRNA[111] and protein has also been reported in normal tissues[112]. In tumors, SOX10 expression has been detected in most MMs and their metastases[113], and in gliomas[114], malignant peripheral nerve sheath tumors[112], clear cell sarcoma[115], invasive breast carcinomas[116] and salivary adenoid cystic carcinomas[117].

Aim of Study

The aim of this study was to investigate SOX10 as a potential biomarker for melanoma and vitiligo.

Summary of Findings

We have successfully established a solid phase proximity ligation assay against the SOX10 protein using polyclonal antibodies. The specificity of the SOX10 assay in serum was high, with only 1% of healthy blood donors exceeding a preset threshold level. In contrast, serum SOX10 levels above this threshold were found in high frequency among vitiligo and melanoma patients. In patients with metastases, lack of SOX10 detection was associated with treatment benefit. In two responding patients, a change from

SOX10 positivity to undetectable levels was seen before the response was evident clinically. We conclude that SOX10 may prove a useful biomarker in diseases of pigmented cells.

Future Perspective

Digital counting of products from detection reactions offers a precise and sensitive way for biomolecule analysis compared to traditional measurements in bulk. The performance of instruments on the market limits opportunities for direct detection of individual biomolecules in recognition reactions. Amplified single molecule detection can serve to simplify instrumentation and improve the detection specificity via the dedicated probing strategy.

RCA based single molecule amplification by sRCA can offer an attractive combination of highly specific detection and prominent amplification together offering single nucleotide discrimination capacity and digital enumeration of reacted probes by flow cytometry via a convenient protocol. The RCA Reporter approach is even more convenient as the second-generation amplification is concurrent with the first generation, but the method may be less suitable than sRCA for purposes of accurately detecting rare single nucleotide variant sequences. Combined with appropriate detection reactions via e.g. proximity reactions (PEA/PLA) or ExCirc and padlock probes, both amplification methods can be applied for detection of either nucleic acid sequences or proteins.

RCA Reporters are typically useful in cases where highly accurate probing is not essential. The single incubation protocol of the RCA Reporters can be applied to detect extension products from proximity assays or other target sequences serving as combined detection and amplification probes. Alternatively, the RCA Reporters can be applied to detect RCA products to enhance the detection signals for flow cytometry based digital counting purpose. It is possible to further increase amplification rate by using two generations of RCA Reporters.

The precise and single nucleotide discrimination capability of sRCA is not limited to the application of rare mutation detection in circulating tumor DNA. Flow cytometric counting of sRCA products from multiplex padlock probe detection reactions of cfDNA renders the technique promising for detection of subtle copy number changes, for example of fetal trisomy in maternal blood. Our data show that coefficients of variation can reach 0.5% when we collect enough events via flow cytometry. If these results will hold up using large panels of padlock probes then we should be able to detect over representation by an extra chromosome from a trisomy fetus with as little as 2% of fetal DNA in cfDNA in maternal blood. This would require to thousands of probes to detect the extra chromosome to even out the probing

efficiency between probes as well as to collect enough events to reach to the CV performance.

Another interesting application of sRCA amplification is multiplex readout by varying the ratio between the colors. We labeled sRCA products using two fluorescent oligonucleotides. By varying the ratio of these two oligonucleotides we were able to distinguish 5 populations of sRCA products in the same plot. Using a library of 3 fluorescent oligonucleotides, we are able to separate 15 distinct populations in 2-color combination. This multiplex strategy can be applied for digital counting of limited panels of biomolecules to facilitate the detection speed as well as the processing workload.

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