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REVIEW

Lock and roll: single-molecule genotyping in situ using padlock probes and rolling-circle amplification

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Abstract In this review I will describe the development of a technique that enables genotyping of individual DNA molecules in the context of morphologically preserved fixed cells, from the fundamental concept published in 1994 to the present status. The review describes enzyme-assisted histochemistry approaches to achieve highly specific molecular identification reactions coupled to efficient signal amplification. The primary molecular identification is accomplished through circularization of oligonucleotide probes, called padlock probes. The circularization reaction is catalyzed by a DNA ligase, which provides robust distinction between single-nucleotide variants under standard reaction conditions. To generate a detectable signal from individual circularized probe molecules, a DNA polymerase is added that replicates probe circles, generating a long tandem-repeated DNA product, easily visualized using a standard epi-fluorescence microscope. Individual signals are recorded as bright dots, providing digital information about the abundance of specific sequences and opportunities for simultaneous detection of several targets using spectral multiplexing. The importance of strictly target-dependent signal amplification will be discussed.

Keywords Padlock probes · Rolling-circle amplification · In situ genotyping · Fluorescence in situ hybridization · DNA ligation

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Introduction

In situ hybridization (ISH) is by far the most important method used to determine the physical location of DNA and RNA molecules in fixed cells. For DNA analysis ISH is most prominently performed using fluorescence-labeled probes (FISH). Localized analysis is important in many fields of research and diagnostics. ISH and FISH are powerful tools for single-cell expression analysis, and FISH is a standard method for analyzing deletions, amplifications and chromosomal translocations in clinical genetics. FISH has furthermore been key to characterize and understand the genetic heterogeneity of tumor material, and it is also widely used for prognostic cancer diagnostics. Traditional ISH techniques have one important limitation, however: they cannot resolve the most common source of genetic variation, namely the millions of characterized single-nucleotide polymorphisms. They further fail to detect all the rare or recurring point mutations occurring in somatic cells, contributing to the development and progression of tumors. In this review, I will describe an approach to extend ISH analyses to also include localized analyses of such common genetic polymorphisms.

Padlock probes

My thesis work concerned the characterization of a new class of oligonucleotide probes for genetic analysis. These so-called padlock probes comprise two target-complementary end-sequences, designed to hybridize head to tail to a target sequence, joined by a target-non-complementary segment. The probes become

circularized by the action of a DNA ligase if they hybridize to the correct target sequence (Nilsson et al. 1994). This novel probe concept was an invention of my thesis supervisor Ulf Landegren and was an extension of the oligonucleotide ligation assay (OLA) that he invented in 1986 and published in 1988 (Landegren et al. 1988).

There are several important properties of the probe circularization concept. First, DNA ligases can very accurately interrogate the identity of the nucleotide complementary to the 3′ end of a probe molecule under standard reaction conditions, because their enzymatic action is very sensitive to mismatches. By designing pairs of probes, differing in the 3′ end only, single-nucleotide polymorphisms can be genotyped by determining which of the probes became ligated (Landegren et al. 1988). Secondly, the circularization reaction is very specific as it can only occur if both probe arms hybridize correctly to the target sequence. The fact that the two probe arms are part of the same molecule makes these probes very useful for highly multiplexed genetic assays because cross-reactive probes generate linear dimeric probes that can be distinguished from the circularized probes created in the correct identification reactions (Banér et al. 2003; Hardenbol et al. 2003, 2005). This property is discussed more extensively in a recent review (Nilsson et al. 2006). Thirdly, upon circularization, the probe molecule becomes topologically locked onto the target molecule, a bit like padlocks to links in a chain (thus the name padlock probe). Finally, the circularized probe can act as a template for a rolling-circle replication reaction (Fire and Xu 1995; Liu et al. 1996).

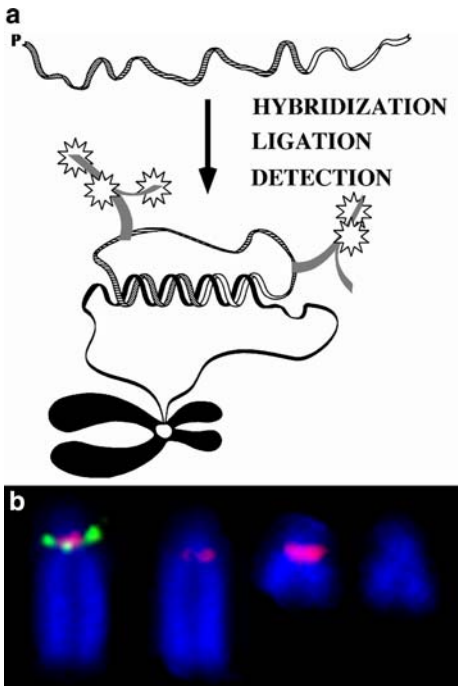

Fig. 1 In situ genotyping of repeated target sequences using labeled padlock probes. **a** Linear padlock probes are hybridized and ligated on denatured metaphase preparations. When hybridized to the correct target sequence, the probes become circularized and topologically locked to the target strand. After stringent washes, the probes are visualized using fluorescence-labeled antibodies. **b** An illustration of the result of an in situ genotyping experiment of a single-nucleotide variation of an alpha-satellite repeat present on human chromosomes 13 and 21. Detection of the two variants gives rise to a *green* or a *red* detection signal. In this particular individual, the green variant was detected on one of the chromosome 13 alleles, the red allele on both chromosome 13 alleles and one chromosome 21 allele, while on one of the chromosome 21 alleles none of the two variants was detected

In situ genotyping of repeated DNA sequences using labeled padlock probes

The first in situ genotyping experiments using padlock probes were achieved in collaboration with Jorn Koch in Aarhus. We targeted a single-nucleotide variation in the alpha-satellite sequence present at the centromeres of human chromosomes 13 and 21 (Nilsson et al. 1997). At that time the sequence variation was thought to be chromosome-specific, but we showed that the two sequence variants could be present on both chromosomes. We used hapten-labeled probes that were visualized using fluorescence-labeled antibodies (Fig. 1). The relative fluorescence signals were so reproducible that we could establish the parental origin of all chromosomes 13 and 21 of an individual after having in situ genotyped the parents and compared the fluorescence profiles of the different chromosome alleles. Here the basis for the allelic resolution of the padlock ligation reaction was the firm association of the circularized probes to the chromosomes, due to the topological lock, which allowed non-ligated probes to be washed away using highly stringent washing conditions.

Together with my student Dan-Oscar Antson, we later extended the repertoire of such allele-specific centromeric probes to chromosomes 7 and 15 (Antson et al. 2003). We further devised a cost-effective PCR-based probe synthesis method that allowed synthesis of much longer padlock probes, incorporating more detection moieties, such as fluorophores and haptens, thus achieving stronger detection signals (Antson et al. 2000). In spite of strong detection signals and the option of applying super-stringent washing conditions to decrease the background, we never succeeded in achieving single-molecule genotyping sensitivity. The reason is most likely that there is no way to distinguish probes that remain in the sample due to proper interac-

tion with the target molecule from probes and detection reagents that stick non-specifically. This effect is nicely illustrated by results obtained during my post-doc at Ton Raap's lab in Leiden. With quite some success we attempted genotyping mitochondrial DNA in situ using labeled padlock probes. But even after careful optimization of the antibody-based signal amplification scheme, we did not manage to obtain perfect distinction between cells carrying two different versions of mitochondrial DNA (Fig. 2). Non-specific sticking of probe and detection reagents is probably impossible to avoid, as a huge molar excess of such reagents has to be added to a sample to drive the detection reactions to completion. Thus, a signal amplification principle should be applied that only gives rise to a strong detection signal if the probes are *correctly* bound.

Rolling-circle amplification

In the mid-1990s, two groups independently found that short DNA circles (26–74 nucleotides long) can template DNA synthesis by DNA polymerases in a rolling-circle replication reaction (Fire and Xu 1995; Liu et al. 1996). Padlock probes are typically between 70 and 100 nucleotides long, so this finding was highly interesting. The observation was somewhat surprising given that the rigid nature of double-stranded DNA prohibits circularization of double-stranded DNA shorter than about 200 bp. Moreover, the size of these small circles is such that a DNA polymerase cannot pass through them. Based on these two facts it can be concluded that: (1) the circular template is only partially double-stranded during replication, which means that the product strand is displaced from the circle ahead of the progressing polymerase; (2) the polymerase does not pass through the circle during replication, which means that the circular template is revolving in two dimensions as the DNA polymerization progresses: around the axis and along the contour of the circle (Fig. 3). As a consequence, the DNA product strand will be displaced at one location and in one direction relative to the polymerase and will never have to be threaded through the circular template (Banér et al. 1998).

In these first studies DNA polymerases with rather low processivity were used, and as it has turned out, they were not very efficient for rolling-circle amplification (RCA). A breakthrough for RCA as an

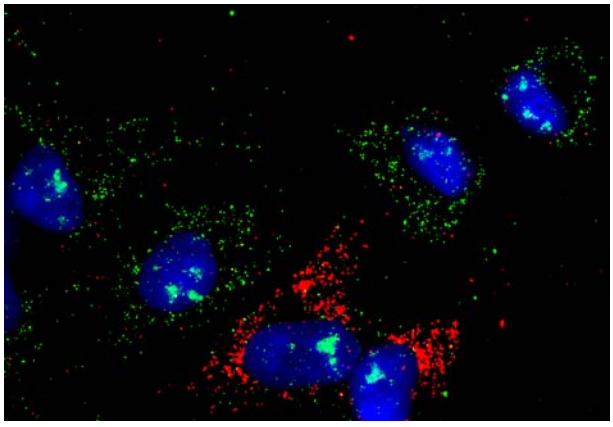

Fig. 2 In situ genotyping of mitochondrial DNA using labeled padlock probes. The A3243G mutation in human mitochondrial DNA was genotyped using the same strategy as shown in Fig. 1. Cell nuclei were DAPI-stained (*blue*) and the two different variant specific probes were visualized using antibodies labeled with *green* (wild-type probe) and *red* (mutant probe) fluorescence. Two different cell lines, carrying either wild-type or mutant mitochondrial DNA, were co-cultured and fixed on microscopy slides. Individual cells from the two cell lines can clearly be distinguished with punctuate signals signifying mitochondrial DNA genotypes. There is also, however, abundant staining of structures in the nuclei, on the slide between the cells, and also to some extent of the wrong genotype in the cytoplasms. These unspecific signals are difficult to avoid as any non-specifically adsorbed probe molecules will give rise to detection signals indistinguishable from correct binding events. The results shown here can be directly compared to the results shown in Fig. 5, as the probes target the same mitochondrial mutation in the same cell lines. The difference is striking and illustrates the importance of a signal generation approach that is absolutely dependent on a target recognition event

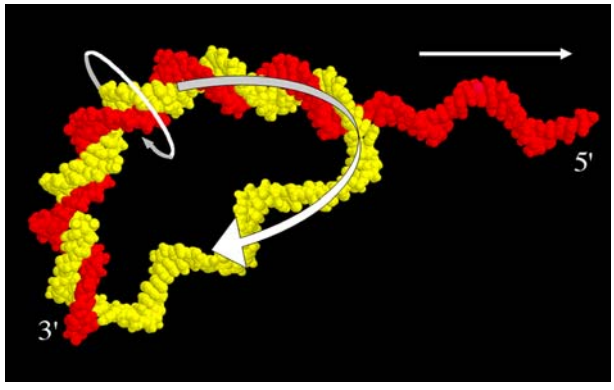

Fig. 3 Rolling-circle DNA synthesis of a small circular DNA template. The picture shows an illustration of how rolling-circle replication of a 70 nucleotide large DNA circle may look like. The DNA polymerase is not shown and would not fit in the picture if drawn to scale. The polymerase adds nucleotides at the 3′ end and thereby induces a rotation of the circle in two dimensions: one, illustrated by the *large bent arrow*, to feed the polymerase with template, and one, illustrated by the *small bent arrow*, around the axis of the template strand as the polymerase cannot pass through the circle. The bend of the DNA duplex is about the maximum bend a double-stranded DNA molecule can have without introducing force, so only a fraction of the circle can be double-stranded at any given point. As a consequence of these restrictions, the product strand should be released at one fixed position relative to the polymerase and in a fixed direction relative to the circle, illustrated by the *straight arrow*. The illustration is a modification of the model made by Johan Banér (1998)

amplification mechanism came when Paul Lizardi introduced the highly processive Φ29 DNA polymerase for RCA (Lizardi et al. 1998). In addition to its processivity, it also has a strong strand displacement activity, as well as strong double- and single-strand 3′ exonucleolytic activity (Blanco et al. 1989; de Vega et al. 1998; Salas 2004). The enzyme produces about 90 kb DNA from each circle per hour, which corresponds to about 1,000 copies of a typical padlock probe (Dahl et al. 2004). Lizardi et al. (1998) showed that individual rolling-circle products could be visualized by hybridizing short fluorescence-labeled detection probes to the tandem-repeated sequence of the product. They further combined padlock probing with RCA, and thus managed to genotype a single-copy gene locus. This early success has been difficult to repeat, however, possibly due to the topological inhibition that we observed and characterized when attempting RCA of padlock probes locked to target molecules (Banér et al. 1998). We found that padlock probes were poor templates for RCA as long as they were attached to long target strands, but that they could be turned into efficient templates if an end was introduced in the target strand close to the probe binding site, e.g., using a restriction enzyme. Once the polymerase manages to displace the probe from the target, the probe can slip off the target strand, templating an unrestrained RCA of the topologically released probe.

Target primed RCA of padlock probes

Christian et al. (2001) suggested using restriction enzymes and 3′ exonucleases to make DNA in cells suitable for padlock probing and RCA. This combination of enzymes had been used before to make target DNA single-stranded for FISH analysis (van Dekken et al. 1988) and could be particularly suitable for padlock probes as the target DNA in the process also becomes fragmented. In this approach a primer was hybridized to the probes to initiate RCA. Again in collaboration with Jorn Koch and Ton Raap, we investigated a very similar approach, but replacing the 3′exonuclease for a 5′ exonuclease. By doing so, a 3′ end is generated in the target strand close to the probe binding site that can act as primer for the RCA (Fig. 4) (Larsson et al. 2004). By using the actual target DNA strand as primer, two important advantages are gained. First, the RCA can proceed in an efficient unrestrained mode, and second, the signal is a covalent DNA extension of the target molecule, which makes the target and signal co-localized per definition.

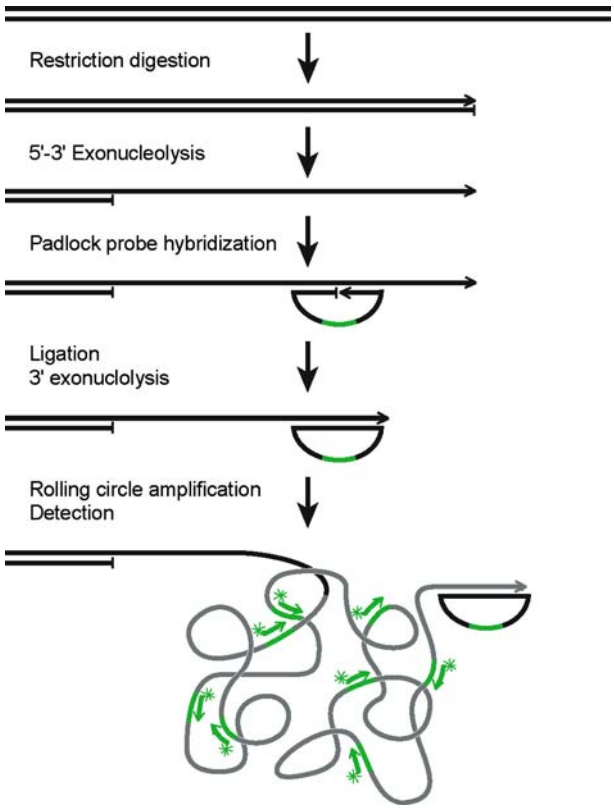

Fig. 4 In situ genotyping through target-primed RCA of padlock probes: an illustration of the different steps of the approach. First the target DNA is restriction digested in situ to introduce an end close to the target sequence. Then the non-target strand is degraded using a 5′ exonuclease, making the target strand available for hybridization. Then the padlock probe is hybridized and ligated. Any 3′ protruding nucleotides will be removed by the strong 3′ exonucleolytic activity of Φ29 DNA polymerase. As the enzyme reaches the probe–target duplex it will initiate a localized amplification of the probes circle, primed by the target strand. Finally, the RCA product is detected using short fluorescence-tagged hybridization probes. The illustration is reproduced from Larsson et al. (2004)

We targeted the mitochondrial point mutation A3243G, causing the rare severe disease MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes, OMIM 540000, Goto et al. 1990) and the more common MIDD (maternally inherited diabetes and deafness, OMIM 520000, van den Ouweland et al. 1992). Due to the maternal transmission of mitochondrial DNA between generations, the high copy number in human cells and segregation to daughter cells by a mechanism fully independent of the spindle apparatus that partitions nuclear chromosomes, the genetics of mitochondrial disorders is quite different from the classical Mendelian inheritance patterns found when mutations affect nuclear genes. The cellular organization of mitochondrial DNA and the mechanisms for its segregation during mitosis and

meiosis are poorly understood, and the present models cannot explain the very uneven distribution of mitochondrial mutation load among members of affected families and between different tissues of affected patients. Mitochondrial DNA is therefore an interesting target for a method that can efficiently score mutation load in individual cells, but is also very useful from a methods development point of view, because effects of optimizations of the experimental procedure result in easily quantifiable responses in the number of signals generated per cell.

In Fig. 5 results are shown, obtained by my student Chatarina Larsson, from an in situ genotyping experiment of the A3243G mutation in two cell lines co-cultured on a microscopy slide. In contrast to the results shown in Fig. 2, the distinction between the two cell lines is almost perfect. In collaboration with Thomas Schmidt in Leiden, we have investigated the nature of the sub-micron sized coils of DNA that are formed in RCA (Blab et al. 2004). We can therefore conclude that the bright dots in the cytoplasms represent detection of single DNA molecules with single-nucleotide selectivity, as they have the same size and intensity as rolling-circle products generated in solution and detected using the same fluorescence-labeled oligonucleotide probes. In Fig. 5, the two different RCA products are distinguished using differentially labeled short fluorescence-labeled hybridization probes that hybridize to sequence motifs that are introduced in the linking segment of the padlock probes. The complements of these sequences are now present in hundreds of copies in the RCA products. Many more padlock probes can be applied and detected using spectrally distinct detection probes, possibly up to around 50, which is the number of different probes that has been resolved in situ using a combined combinatorial and ratio labeling scheme (Brink et al. 2002; Tanke et al. 1999). The steps in the procedure are probably driven close to completion by applying relatively high concentration of probes and enzymes. This can be achieved without introducing detectable background noise due to the specificity of the probe circularization approach coupled to the circle-specific signal amplification. Because of the high concentrations used, the incubation time in each step can be kept short, which means that results can be obtained in less than 3 h after fixation of the cells. Furthermore, the technique is more robust and has successfully been applied by close to a hundred students, inexperienced in histochemistry or FISH techniques, during several EMBO practical training courses in Uppsala.

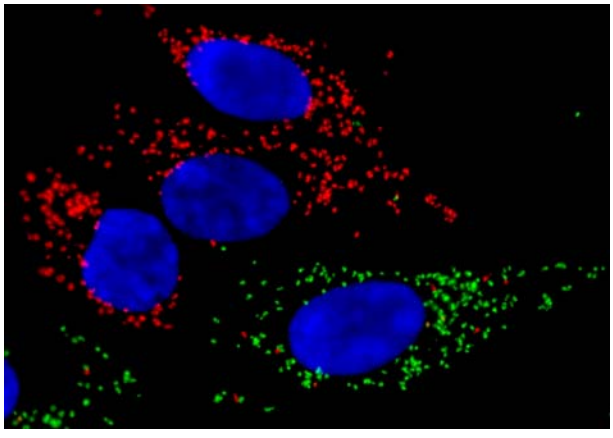

Fig. 5 In situ genotyping of mitochondrial DNA using target-primed RCA of padlock probes. The same mitochondrial mutation was targeted in the same cell lines as the one in Fig. 2, but now using padlock probe and the target-primed RCA approach outlined in Fig. 4. Cell nuclei were DAPI-stained (*blue*) and the RCA products generated from the wild-type and mutant probes were visualized using red and green fluorescence-labeled oligonucleotides, respectively. Virtually all signals reside in the cytoplasms, compared to the noisier staining pattern in Fig. 2. The signal intensity and signal to background ratio is much higher, typically allowing for ten times shorter exposure times. The rare mutant signals (*green*) in the wild-type cells probably represent mis-ligation events, while the wild-type signals (*red*) in the mutant cells may be true detection signals as there is no way to select for 100% mutant cells

Future perspectives

We have recently, in collaboration with Andrew Collins in Oslo, applied our in situ genotyping technique to detect mitochondrial DNA and nuclear Alu-repeat sequences in so-called comet preparations used to study DNA damage and repair (Shaposhnikov et al. 2006). We are now adapting the technique to allow analysis of single-copy nuclear genes (Henriksson et al., in preparation) and their transcripts (Larsson et al., in preparation) in fixed cells and in tissue sections of samples where we expect genetic heterogeneity, such as clinical tumor material. With this in situ genotyping method we aim to enable efficient detection of deletions and multiplications of genes with allelic resolution, studies of the distribution of point mutations, X-inactivation patterns and allelic imbalances of transcripts.

Acknowledgments The results I have described have been achieved through the work of many collaborators and co-workers. I would particularly like to emphasize the contributions of Ulf Landegren, Jörn Koch and Ton Raap, as well as the former and present students Johan Banér, Dan-Oscar Antson, Chatarina Larsson and Sara Henriksson. I further thank Landegren and Raap for valuable comments about the manuscript. My work has been supported by grants from the Biejer, Linné and Wallenberg

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