In situ genotyping individual DNA molecules by target-primed rolling-circle amplification of padlock probes

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Methods are needed to study single molecules to reveal variability, interactions and mechanisms that may go undetected at the level of populations of molecules. We describe here an integrated series of reaction steps that allow individual nucleic acid molecules to be detected with excellent specificity. Oligonucleotide probes are circularized after hybridization to target sequences that have been prepared so that localized amplification reactions can be initiated from the target molecules. The process results in strong, discrete detection signals anchored to the target molecules. We use the method to observe the distribution, within and among human cells, of individual normal and mutant mitochondrial genomes that differ at a single nucleotide position.

By analyzing biological processes at the ultimate level of single molecules, and with sufficient precision to distinguish even closely similar variants, it will be possible to study the inter- or subcellular context of specific DNA or RNA sequences as well as to analyze their location among extensive, arrayed sets of biological samples. Unlike simple hybridization probes, PCR provides adequate specificity to detect unique target sequences in the context of whole genomes, but the technique is poorly suited for precisely localized detection reactions. Oligonucleotide probes with one target-complementary sequence at each end—padlock probes—encircle specific target molecules as a consequence of templated DNA ligation. These probes have detection specificity similar to that obtained with PCR[1–3], but unlike in PCR, the reaction products can remain bound to their target sequences, even withstanding denaturing washes[4]. Unfortunately, detection signals from specifically circularized padlock probes have proven insufficient to reveal single-copy gene sequences owing to background noise from nonspecifically bound probes, although repeated centromeric sequences have been evaluated with sufficient precision to investigate the distribution of single-nucleotide variants *in situ*[5,6].

Linear oligonucleotide probes hybridized *in situ* have been used as primers for site-specific DNA synthesis, allowing labeled nucleotides to be incorporated in the so-called PRINS reaction[7]. This process enables localized allele-specific detection of repeated sequences in the genome[8–11], but this method, too, falls short of detecting single-copy genes from single primers, as would be needed for genotyping *in situ*. More recently, linear probes hybridized *in situ* have been extended not by replicating the target sequence, but by using separately added small circular DNA strands as templates for a localized rolling-circle amplification (RCA)[12,13]. Although this results in clearly detectable signals from individual probes, the specificity of the reaction may be insufficient for robust detection of unique single-copy sequences *in situ* because of difficulties in distinguishing specifically bound from nonspecifically bound probes.

If, instead, the reaction is set up so that the RCA template can form only in a target-specific circularization reaction *in situ*, nonspecific signals should be greatly diminished; and promising results have been reported with this approach[14,15]. Regrettably, replication of circular probes that remain wound around their target strands seems to be inhibited, probably as a consequence of molecular crowding[16], although there is some controversy in the literature about this topological inhibition[17]. In an interesting approach, target sequences were enzymatically rendered single stranded having free 5′ ends near the binding sites for padlock probes[15]. Upon addition of a primer the probes can be replicated *in situ* by RCA, but only if they escape the topological block against replication by detaching from the target molecules. In the process, amplification products risk drifting away, however, potentially further reducing the efficiency of localized detection.

We now present a procedure that integrates highly specific and sensitive recognition of target nucleic acid sequences using padlock probes with efficient local signal amplification, thereby permitting detection of single-nucleotide variation of individual DNA molecules *in situ*. Padlock probes, circularized in a target-dependent manner, serve as templates for uninhibited rolling circle reactions primed from the actual target sequences. In this way, the anchored target molecules become extended by hundreds of copies of the reacted probes, readily available for detection using standard

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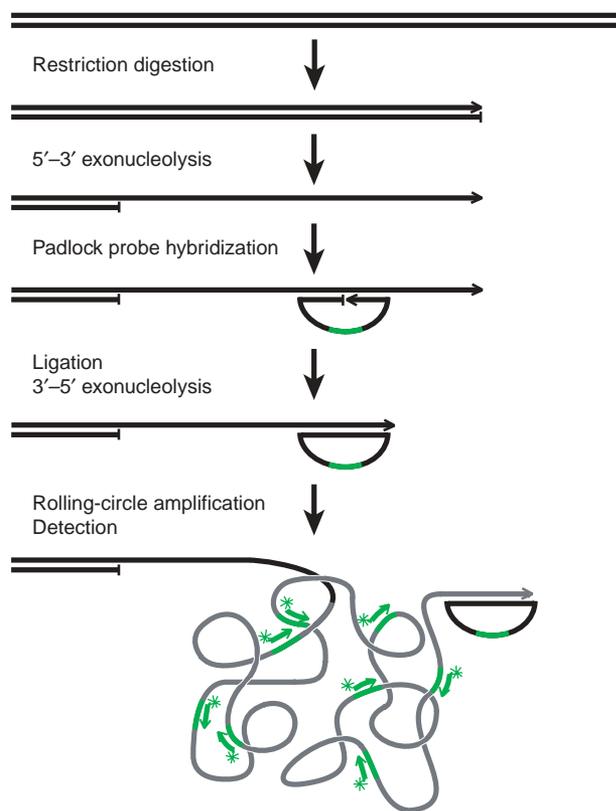
Figure 1 | Schematic representation of target-primed rolling-circle amplification of circularized padlock probes. The target DNA is restriction digested 3′ of the target sequence and irreversibly made single stranded by strand-specific 5′–3′ exonucleolysis. Padlock probes are hybridized to their target sequences and the probe ends are joined through ligation, locking the probe onto the target molecule. After ligation, the RCA is initiated by the Φ29 DNA polymerase by turning the target molecule into a primer through 3′–5′ exonucleolysis of any 3′ end protruding beyond the padlock probe hybridization site. The padlock probe then serves as the template for DNA synthesis. The RCA product (gray) is detected through hybridization of fluorescence-labeled oligonucleotides to tag sequences (green) specific for the padlock probe. Arrowheads indicate 3′ ends and bars 5′ ends.

Figure 2 | Assessment of enzymatic target preparation of mtDNA in cultured cells. Nuclei were stained with DAPI (blue) and digoxigenin-labeled padlock probes hybridized and ligated to the target sequence were visualized by immunohistochemistry (red). A map of the target and restriction sites is shown above the images, and insets show the expected configurations of the target fragments. The sense strand is shown in yellow and the antisense strand in white. Arrowheads in insets indicate 3′ ends and bars 5′ ends. The cells shown in the left column were restriction digested 5′ of the target sequence using *Eco*RV, and the cells shown in the right column were digested 3′ of the target sequence using *Msc*I. The cells shown in the top row were treated with a 5′–3′ exonuclease (λ exonuclease), and the cells shown in the bottom row were treated with a 3′–5′ exonuclease (exonuclease III) after restriction digestion. Scale bar, 20 μm.

hybridization probes. We compared our method to other approaches, and used it to observe the distribution of normal mitochondrial genomes and ones harboring a single-nucleotide variant, characteristic of the MELAS syndrome, in homo- and heteroplasmic cell lines and in histological tissue sections.

RESULTS
Method Design
Normal and mutant mitochondria often coexist in the cells of patients with mitochondrial disease, a phenomenon termed heteroplasmy, and the proportions can shift markedly from mother to offspring or between somatic cell generations[18]. Here we have designed padlock probes to genotype the mitochondrial A3243G mutation *in situ*. This mutation is observed in the severe but rare MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes, OMIM 540000)[19] and in the more common MIDD syndrome (maternally inherited diabetes and deafness, OMIM 520000), which may account for as much as 1% of cases of diabetes[20].
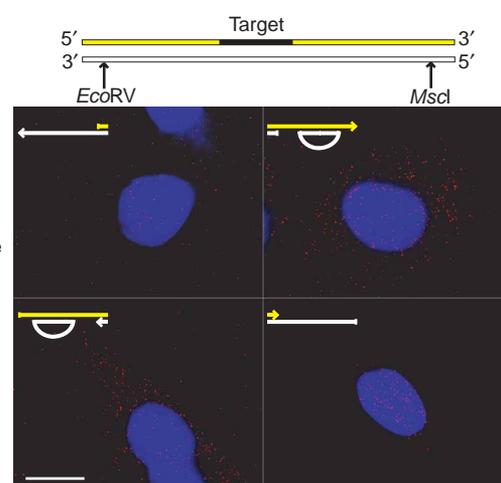
By amplifying circularized padlock probes through rolling circle DNA synthesis *in situ*, specifically reacted probes can be distinguished from nonspecifically bound probe molecules and other detection reagents. We modified an enzymatic target preparation procedure previously used for *in situ* amplification of padlock probes[15] to produce single-stranded target strands with free 3′ ends downstream of the recognition sequences for the padlock probes, allowing target-primed amplification of circularized probes. Thus, the target strand can first act as the template for circularization of the probes and then prime RCA, thus avoiding topological inhibition of replication and ensuring efficient retention of the amplification products at the original location of the target sequences (**Fig. 1**). Any 3′ end sequence of the target strand that protrudes beyond the probe-recognition site can be removed by the 3′–5′ exonucleolytic activity of the Φ29 polymerase[21], until the enzyme can use the padlock probe to template the RCA reaction. The RCA product, continuous with the target strand, consists of hundreds of single-stranded copies of the probe, repeated in tandem and available for detection by fluorophore-labeled hybridization probes directed towards tag sequences included in the non-target-complementary segment of the padlock probes[22].

Mechanistic studies
We evaluated the efficiency of the individual reactions steps of the integrated procedure in two separate series of experiments. First, the enzymatic target preparation procedure was investigated using different combinations of restriction enzymes and exonucleases to expose either the sense- or the antisense strand at the A3243G *MELAS* locus of the mitochondrial genome. The targets were
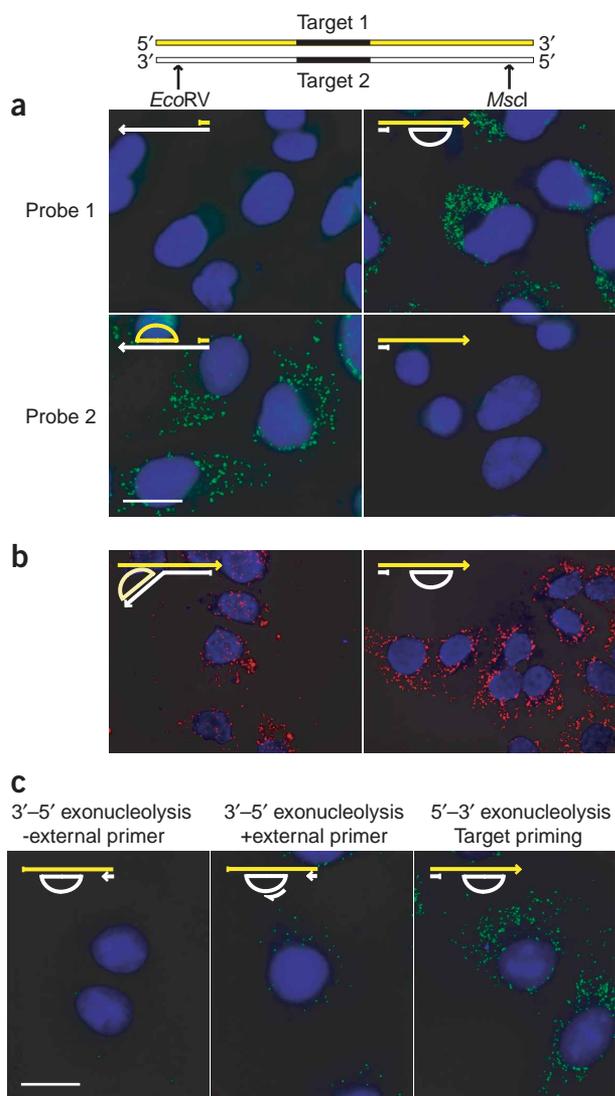
Figure 3 | Target-primed RCA of padlock probes. Nuclei are shown in blue and RCA products in green or red. A schematic representation of the target- and restriction sites is shown on top of the images. Expected configurations of the target fragments are shown as insets. The sense strand is shown in yellow and the antisense strand in white; 5′ ends are indicated with bars and 3′ ends with arrowheads. Scale bars, 20 μm. (**a**) The two strand-specific padlock probes, 1 (ppMUTs; top row) and 2 (ppMUTas; bottom row), were amplified by RCA after target recognition. The mitochondrial genome in the M50 cell line was digested with either *Eco*RV (left) or *Msc*I (right), making one or the other strand of the target locus single stranded after λ exonuclease treatment. (**b**) Left, results from hybridization of a linear oligonucleotide probe to the target, with amplified detection through RCA of a preformed DNA circle. Right, results from target-primed RCA of the padlock probe ppWTs, detecting the same sequence as the linear probe. The target sequence in the W7 cell line was rendered single stranded by treatment with *Msc*I and λ exonuclease. (**c**) The target strand for probe 1 was prepared single stranded with either a free 5′ or a free 3′ end. From left, the images show results from M50 cells displaying a free 5′ end of the target strand without and with added primer Lin33c, and from cells displaying a free 3′ end without added primer.

MELAS locus (ppMUTs) and one specific for the antisense strand (ppMUTas). Cells grown and fixed on slides were subjected to restriction digestion either 5′ or 3′ of the target sequence, followed by degradation of the 5′ ends using λ exonuclease to expose the probe recognition sequences. This produced single-stranded sense or antisense target sequences with nearby free 3′ ends that could potentially prime RCA of reacted probes (**Fig. 1**). Signals were obtained for both the sense- and the antisense probes in samples treated to prepare the appropriate target sequences, and no signals were seen when padlock probes were combined with target sequences of the wrong polarity (**Fig. 3a**). The signals appeared as multiple brightly fluorescent spots with diameters of about 0.5 μm, probably representing individual rolling circle products, as sizes and signal intensities were similar to those of RCA products formed in solution and deposited on glass slides (data not shown). Larger signals of variable morphology may correspond to several adjacent rolling circle amplification products. The strength of the signals allowed a tenfold shorter camera exposure time to be used as compared to when padlock probes were detected with antibodies, and resulted in far greater contrast between signals and the background from autofluorescence.

We carried out experiments to compare the present method to previously published *in situ* RCA procedures[12,15]. The first strategy used preformed circular DNA strands to template RCA reactions *in situ* to detect the hybridization of sequence-specific linear probes to their target sequences[12]. To compare this strategy with ours, we used a 70 nucleotide (nt) linear probe (3243CMV), the 40 5′-most nucleotides of which were designed to hybridize to the wild-type variant at the *MELAS* locus, whereas the 30 nt at the 3′ end were complementary to the padlock probe ppCMV93. The padlock probe was hybridized to the linear hybridization probe and converted to a circle by ligation. The linear probe and the circle were added to the slides and allowed to hybridize, and then washed as described in the original protocol[12]. The slides revealed cytoplasmic signals, but also abundant signals inside nuclei and outside cells, in contrast to the target-primed RCA experiment done in parallel where signals were confined to the cytoplasm (**Fig. 3b**). Similar results were obtained whether target DNA was rendered single stranded by enzyme treatment or by heat denaturation.

probed using the digoxigenin-labeled padlock probe ppMUT-DIG, which is specific for the sense strand, and these probes were then detected using fluorescent antibodies against digoxigenin (**Fig. 2**). This is in contrast to all subsequent experiments, in which unlabeled probes were rolling-circle amplified for enhanced detection. By using a direct labeling system, the target preparation method could be demonstrated independently of the target-primed RCA mechanism. Despite considerable nonspecific background, cytoplasmic signals indicating the presence of mitochondrial genomes were clearly more abundant under those two experimental conditions where the correct target strand was prepared for hybridization. The number of signals, and the nonspecific background, were similar when target sequences were prepared by standard chemical denaturation with 50% formamide in 2× SSC at 80 °C for 90 s (data not shown). By contrast, combinations of enzymes that exposed the nontarget strand resulted in only nonspecific background signals (**Fig. 2**), similar to results obtained when no restriction or exonuclease digestion was undertaken (data not shown).

Next, we investigated under what conditions *in situ*–circularized probes could be amplified by an RCA procedure. For this purpose we used one padlock probe specific for the sense strand at the
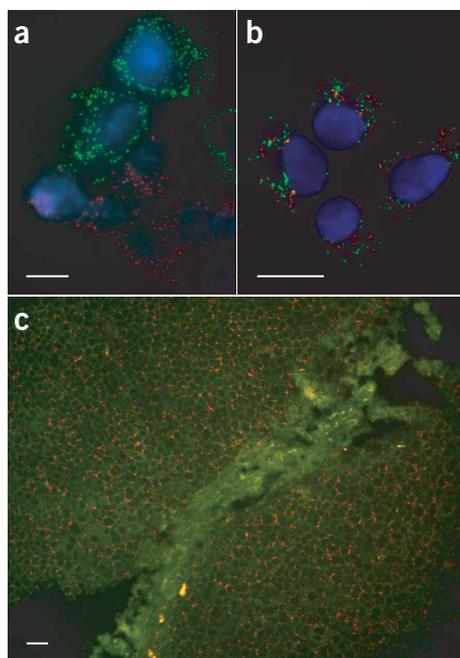
Figure 4 | *In situ* genotyping of the mitochondrial A3243G point mutation. We applied the target-primed RCA method to detect two padlock probes, each specific for one of the two single-nucleotide variants of the target sequence. Signals from the two probes are shown in red (ppWTs) and green (ppMUTs) and the DAPI-stained cell nuclei are shown in blue. Scale bars, 20 μm. (**a**) Genotyping of the W20 and M50 cell lines, homoplasmic for the two different genotypes, *in vitro* cultured together on a slide. (**b**) Genotyping of the heteroplasmic G55-1.1 cell line. (**c**) Genotyping of mitochondrial genomes in a tonsil tissue section expected to contain only the wild-type target sequence. We subjected the tissue section to the same target-primed RCA procedure as the *in vitro*–cultured cell lines.

night, but this did not give rise to detectable signals in our hands (data not shown).

We further examined how the size of the target fragment affects the detection efficiency. By using target sequences located on different target fragments generated by the same restriction enzyme, we found that a 2,992 base pair (bp) restriction fragment yielded five times more signals than a 534 bp fragment, probably reflecting loss of some of the shorter fragments after restriction digestion (see **Supplementary Note** and **Supplementary Fig. 1** online). We also investigated the ability of the Φ29 DNA polymerase to degrade 3′ end sequences until they can serve as primers for the RCA reaction. When target sequences were prepared having free 3′ ends at different distances from the site of probe binding, we found that a 3′ end located 130 nt downstream of the circularized probe was detected with half the efficiency compared to a target where the 3′ end was located immediately downstream of the probe-binding site (see **Supplementary Note**, **Supplementary Fig. 2** and **Supplementary Table 1**).

In situ genotyping of mitochondrial genomes

We used a pair of padlock probes specific for the two sequence variants to genotype the mitochondrial A3243G point mutation *in situ* in three cell lines. Two cell lines that were homoplasmic for either mitochondrial sequence variant were cocultured, fixed on a glass slide and then subjected to target-primed RCA of *in situ*–circularized probes. RCA products were detected by hybridization of two fluorescent oligonucleotide probes with sequences identical to distinct tag sequences in the two variant-specific padlock probes (**Fig. 4a**). The single-nucleotide difference between mitochondrial

Next, we compared our procedure with a strategy where target sequences with nearby 5′ ends were obtained by enzyme treatment and padlock probes were amplified using an added oligonucleotide primer[15]. In this comparison we added the ppMUTs probe to samples where restriction digested target sequences had been made single stranded using exonuclease III, producing a free 5′ end upstream of the probe recognition site. When no primer was added, almost no signals appeared, because no 3′ ends capable of priming RCA were created. When a primer complementary to the padlock probe was added to such reactions, about tenfold more signals were detected. The efficiency was still at least ten times lower, however, than when the same probe was used to detect target sequences prepared to have nearby free 3′ ends, allowing signals to be amplified by target-primed RCA (**Fig. 3c**). We used the Φ29 DNA polymerase to perform the RCA in these experiments. To reproduce the conditions of the original publication[15], we also carried out the RCA with ThermoSequenase over

Table 1 | Padlock probes and detection probes

Probe	Sequence
ppMUT-DIG	5′-P-*CTGCCATCTTAACAAAC*TTTCTCTTTATG(DIG)CTTAGTGACCTGCAG(DIG)TATTTCTCTTT*CGATTACCGGGCC*-3′
ppWTs	5′-P-*CTGCCATCTTAACAA*TTCCTTTTACGACCTCAATGCTGCTGCTGTACTACTCTTC*TGCGATTACCGGGCT*-3′
ppMUTs	5′-P-*CTGCCATCTTAACAA*CCTTTCCTACGACCTCAATGCACATGTTTGGCTCCTCTTC*TGCGATTACCGGGCC*-3′
ppMUTas	5′-P-*GCCCGGTAATCGC*CTTTCACGACCTCAATGCACATGTTTGGCTCCGCGACTAT*TTTGTTAAGATGGCAGG*-3′
ppMSCas	5′-P-*CCTCATTGTACCCATT*TTTCTACGACCTCAATGCTGCTGCTGTACTACTCTTCTAT*GCCAACCTCCTACT*-3′
ppMSCs	5′-P-*TAAGAAGAGGAATTG*CCTTTCCTACGACCTCAATGCACATGTTTGGCTCCTCTTC*CCATGGGTATGTTGT*-3′
ppCMV93	5′-P-*CCTCCCATCATATTA*AAGGCTTTCTCTATGTTAAGTGACCTACGACCTCAATGCTGCTGCTGTACTACTCTTCCTAAG*GCATTCTGCAAACAT* -3′
3243CMV	5′-P-TATGCGATTACCGGGCTCTGCCATCTTAACAAACCCTGTTTAATATGATGGGAGGATGTTTGCAGAATGC-3′
Lin33c	5′-GGAGCCAAACATGTGCATTGAGG-3′
Lin16	5′-Cy3-CCTCAATGCTGCTGCTGTACTAC-3′
Lin33	5′-FITC-CCTCAATGCACATGTTTGGCTCC-3′

The padlock probes ppMUT-DIG and ppCMV93 were kindly provided by Eurogentec. The other padlock probes, the Lin33c primer and 3243CMV were purchased from DNA Technology A/S. The Lin16 and Lin33 fluorescence-labeled probes were purchased from Thermo Hybaid. P, 5′ phosphate; DIG, digoxigenin. Target-complementary sequences of padlock probes are shown in italics and tag sequence segments of padlock probes used for fluorescence detection are color coded.

genomes in the two cell lines was clearly visualized, with one class of cells showing predominantly green signals and the other one red signals. The strong, discrete signals also allowed the distribution of the two mitochondrial genome variants to be studied within cells of a heteroplasmic cell line (**Fig. 4b**). By counting red and green fluorescence signals in 31 cells, we estimated the average heteroplasmy level as 66% ± 8% (mean ± s.d.) mutant mitochondrial DNA molecules in this cell line, in good agreement with the result of 64% ± 7% obtained for the same cell line using gel-based quantification[23] (see **Supplementary Fig. 3** and **Supplementary Methods** for details). We genotyped the same locus in a tonsillar tissue section, demonstrating that the fluorescence from individual amplification products stands out prominently from any autofluorescence in this tissue (**Fig. 4c**). Only wild-type signals were seen in the sample we investigated, which is expected considering the low frequency of the mutation in the general population[24].

DISCUSSION

We introduce here a procedure for analyzing individual DNA molecules *in situ* by target-primed RCA of *in situ*–circularized padlock probes, with specificity sufficient to resolve single-nucleotide variation. This subtle target variation is converted into distinct tag sequences in the RCA products that are easily decoded by low-stringency hybridization of a standard pair of color-coded oligonucleotide detection probes. Because the localized signal amplification strictly requires a sequence-dependent padlock probe circularization, there is no background arising from non-specifically bound probes and thus stringent or extensive washes are not required. Furthermore, samples are never exposed to temperatures over 37 °C and they can be fixed by mild ethanol treatment, preserving cellular morphology.

We applied our method to study sequence variation in the mitochondrial genome, enabling analysis of the distribution of mitochondrial genomes within cells. The overall detection efficiency was estimated to be about 10% of all potential target sequences in the cells, based on the number of mitochondrial genomes in the cells as determined by real-time PCR (data not shown). We estimated the proportion of mutant and wild-type target sequences in individual heteroplasmic cells by counting individual amplification products, and the average result of this digital measurement was in good agreement with the result from standard PCR estimation at the population level. A detailed analysis of the intracellular distribution of mutant and wild-type mitochondrial genomes will be presented elsewhere (M.N., Karoly Szuhai, Hans Vrolijk, G.J., J. Antonie Maassen, A.K.R. *et al.*, unpublished data). The present method allows, for the first time, robust analysis of the inter- and intracellular distribution of mitochondrial single-nucleotide DNA sequence variants. Our molecular approach for robust, specific analysis of single DNA molecules should also be applicable to localized detection of DNA sequences in other contexts, including DNA molecules located in the cytoplasm of cells, such as episomal viral or bacterial genomes, and *in situ* reverse-transcribed cDNA. The current detection efficiency of 10%, however, precludes the use of the method for genotyping of nuclear single-copy genes in single cells.

Target-primed RCA of *in situ*–reacted padlock probes is a conceptually attractive procedure, combining highly specific detection with an essentially background-free amplification step. We compared this procedure experimentally to previously described procedures for DNA detection *in situ*. In our hands,

amplification of preformed DNA circles[12] resulted in unacceptably high background, precluding detection of low-copy-number target sequences (**Fig. 3b**). Padlock probes that form circles only upon target recognition provide greater specificity of detection. We found that RCA templated by padlock probes that remain bound to their target sequences is relatively inefficient if an external primer is used[15], probably because of steric hindrance[16]. As reported herein, we overcame this problem by arranging for the target molecule to serve as the amplification primer, which has the added benefit that the amplification products remain firmly anchored.

In conclusion, target-primed RCA of *in situ*–reacted padlock probes allows specific nucleic acid sequences to be investigated in cells and in tissue preparations. By identifying individual target molecules, digital information about their abundance and location can be obtained. The method permits studies of mitochondrial DNA organization that may provide fundamental insights into the pathogenesis of mitochondrial disease. It may also find applications in *in situ* studies of infectious organisms, and in the analysis of expressed members of conserved gene families, splice variants and allelic sequence variants, as well as arrays of samples, both in research and in routine pathological examinations.

METHODS

Sample pretreatments. The M50, W7, W20 and G55-1.1 cell lines[23] were cultured and fixed on glass slides (**Supplementary Methods**). We prepared cells for *in situ* analyses by treatment with 0.01% pepsin (Sigma) in 0.1 M HCl for 90 s at 37 °C, followed by brief washes in PBS. Anonymous tonsil tissue sections, fresh frozen and fixed in acetone, were obtained from the Department of Genetics and Pathology at the Uppsala University Hospital. Target-primed RCA of tissue samples was carried out as described for the cultured cells, except that the pepsin treatment was extended to five minutes.

We carried out the following reactions in a 50 µl reaction volume under a 24 × 55–mm cover slip, or in reaction chambers defined by a silicone mask placed directly onto the slides, prepared essentially as previously described[25] (for details see **Supplementary Methods**).

Enzymatic target preparation. We made target sequences accessible for hybridization by digestion with a combination of restriction and exonuclease enzymes[26]. The DNA was first restriction digested using 0.5 U/µl of *Msc*I (New England Biolabs) or *Eco*RV (AP Biosciences) at 37 °C for 30 min in the corresponding supplied buffers supplemented with 0.2 µg/µl BSA (New England Biolabs). We then rinsed the slides in buffer A (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl and 0.05% Tween-20). We made the ends of the restriction fragments single-stranded by exonucleolysis, using 0.2 U/µl of either the 3′–5′ exonuclease III (New England Biolabs) or the 5′-3′ λ exonuclease (New England Biolabs), at 37 °C for 15 min in the corresponding supplied buffers supplemented with 0.2 µg/µl BSA and 10% glycerol. After incubation the slides were rinsed in buffer A.

Padlock probe hybridization and ligation. Oligonucleotide sequences are presented in **Table 1**. For direct detection of padlock probes, we hybridized 20 nM of the padlock probe ppMUT-DIG in a solution of 2× SSC, 20% formamide and 0.5 µg/µl sonicated salmon sperm DNA at 37 °C for 15 min. For RCA detection, we hybridized 100 nM of padlock probe under the same conditions as

for direct detection. We removed excess probe by a wash in buffer B (2× SSC, 0.05% Tween-20) for 5 min at 37 °C and then a rinse in buffer A. Padlock probes were circularized in 10 mM Tris-acetate pH 7.5, 10 mM MgAc$_2$, 250 mM NaCl, 1 mM ATP, 0.2 µg/µl BSA and 0.1 U/µl T4 DNA ligase (Amersham Biosciences) at 37 °C for 15 min. If the padlock probes were not detected through RCA, we washed the slides stringently and visualized bound probe with two layers of fluorescence-labeled antibodies (**Supplementary Methods**). Slides for RCA detection were washed once in buffer B for 5 min at 37 °C, rinsed once in buffer A and dehydrated in a series of 70%, 85% and 100% ethanol.

Rolling circle amplification.[14,16,27,28] We found that the use of rubber silicone reaction chambers greatly improved the efficiency of the RCA reaction. Therefore, we performed the RCA reactions on dehydrated slides in 50 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 20 mM (NH$_4$)$_2$SO$_4$, 0.2 µg/µl BSA, 1 mM DTT, 0.25 mM dNTP, 10% glycerol and 1 U/µl Φ29 DNA polymerase (Fermentas) at 37 °C for 30 min in the silicone mask reaction chambers placed on a shaking platform, except for the tonsillar tissue section, where the RCA was carried out under a coverslip. After polymerization, we removed the masks and rinsed the slides in buffer A. We detected the single-stranded RCA products by hybridizing 250 nM of Lin16 and Lin33 fluorescence-labeled oligonucleotide probes in a solution of 2× SSC, 20% formamide and 0.5 µg/µl salmon sperm DNA for 15 min at 37 °C. After a brief rinse in buffer A, we finally dehydrated the slides, mounted them in Vectashield (Vector Laboratories) containing 10 ng/ml DAPI and stored them at 4 °C. Experimental details of the comparisons with related methods are described in **Supplementary Methods**.

Image analysis. We acquired Images using an epifluorescence microscope (Axioplan II, Zeiss), equipped with a 100 W mercury lamp, a CCD camera (C4742-95, Hamamatsu) and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC and Cy3. A 100× objective (Plan-neofluar, Zeiss) was used for all images, except in **Figure 4a** (63×, Plan-neofluar) and **Figure 4c** (20×, Fluar), and images were captured using 0.2 s exposure time. We collected images using the Imstar software (Imstar) and thresholded them in Adobe Photoshop Elements 2.0 (Adobe Systems).

Note: Supplementary information is available on the Nature Methods website.

COMPETING INTERESTS STATEMENT
The authors declare competing financial interests; see the *Nature Methods* website for details.

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