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(54) Benævnelse: **Fremgangsmåde, sonde og kit til in situ-hybridisering af DNA og anvendelse deraf**

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DESCRIPTION

[0001] The invention is directed to a method for analyzing events associated with replication in the genomic DNA in a eukaryotic cell, especially a mammalian cell, using *in situ* hybridization techniques. Is disclosed herein a method especially directed to the detection of the occurrence of initiation of replication events in genomic DNA, in which the co-detection of DNA and RNA molecules in a single cell is enabled. According to a further particular aspect, the co-detection and co-visualisation of DNA and optionally RNA and protein(s) in a single cell is enabled.

[0002] In the context of the present disclosure, a genomic DNA molecule is a nucleic acid molecule that belongs to the genome of a cell, and replicates, especially in an autonomous or independent manner, in particular under the control of cellular regulatory elements, in metabolically active cell(s), and whose replication can therefore be observed *in situ*. The genome of a cell is considered to be the DNA of an organism, that carries all the information for all the proteins the organism will ever synthesize, and more generally the genome contains all the information necessary for the survival of a cell. Genomic DNA can be chromosomal DNA or plasmidic DNA, with the proviso that said plasmidic DNA belongs to the genome of a cell, as defined herein. More particularly in the context of the present disclosure, genomic DNA (gDNA) molecule(s) is either mitochondrial gDNA or nuclear gDNA or both. A plasmid is a DNA molecule that is separate from, and can replicate independently of, the chromosomal DNA. Plasmids are double stranded and, in many cases, in particular in most cases, circular. Plasmids usually occur naturally in bacteria, but are sometimes found in eukaryotic organisms. By contrast, plasmidic DNA not belonging to a genome as functionally defined is not considered to be genomic DNA in the context of the present disclosure.

[0003] The invention also relates to specific probes, in particular nucleotide probes, which are particularly devised for the detection of the occurrence of initiation of replication events in genomic DNA in a eukaryotic cell. The invention encompasses means useful for detecting the occurrence of initiation of replication events in genomic DNA, in particular kits comprising such probes and processes for carrying out the invention.

[0004] According to particular embodiments, the methods, probes and kits of the invention are suitable for analyzing initiation of replication of genomic DNA at the single cell level, and therefore provide means for detecting impaired replication of gDNA, and in particular means useful for detecting diseases associated with such impairment, including mitochondrial disease(s), neoplasic diseases(s) or cancer(s).

[0005] In a particular embodiment, the invention especially relies on the results obtained in experiments designed to observe the occurrence of initiation of replication events in mitochondrial genomic DNA in human cells.

[0006] Indeed, mitochondrial DNA (mtDNA) replication and transcription are crucial for cell function, but these processes are poorly understood at the single-cell level. Tools currently offered to biologists do not permit the specific detection of mitochondria engaged in initiation of DNA replication. With respect to nuclear gDNA, tools currently offered to biologists do not permit the detection of nuclear gDNA engaged in initiation of its replication within a cell, i.e. at the single cell level, not isolated from its cellular context.

[0007] Mitochondria are energy-producing organelles whose function is directed not only by the nuclear genome but also by their own genome. Each mitochondrion carries several copies of a genomic DNA, i.e. a circular double-stranded DNA that is replicated and transcribed autonomously in the organelle. Human mtDNA, a 16.5 kbp molecule, is organized in 13 protein-coding, 2 rRNA, and 22 tRNAs genes that are transcribed from the (heavy) H-strand (12 mRNA, 2 rRNA and 14 tRNAs) and from the (light) L-strand (1 mRNA for *ND6* gene, and 8 tRNA) with production of polycistronic precursor RNAs. These primary transcripts are processed to produce the individual mRNA, rRNA and tRNA molecules [1]. The prevalent view of mtDNA replication is that DNA synthesis starts from origin O_H where the nascent H strand frequently terminates 700 bp downstream giving rise to the 7S DNA, which produces a characteristic triple stranded structure, the D-loop[2,3]. When leading strand synthesis has reached two thirds of the genome, it exposes another major origin, the origin of L-strand DNA replication (O_L), and lagging-strand DNA synthesis then initiates in the opposite direction. Conversely, coupled leading and lagging strand synthesis has been described in a reduced number of molecules[4].

[0008] Nuclear DNA (nDNA) is generally compacted in chromosome(s), and its replication generally begins at specific location(s) in the genome, called "origin(s)" or "replication origin(s)", which is/are the positions at which the DNA helix is first opened, giving rise to a "replication bubble". Unwinding of DNA at the origin, and synthesis of new strands, forms a replication fork, which has an asymmetric structure. The DNA daughter strand that is synthesized continuously is known as the leading strand, whose synthesis slightly precedes the synthesis of the daughter strand that is synthesized discontinuously, known as the lagging strand.

Eukaryotic chromosomes generally contain multiple origins of replication. The different replication origins in eukaryotic chromosomes can be activated in a sequence, determined in part by the structure of the chromatin, with the most condensed regions of chromatin beginning their replication last.

[0009] The processing of mitochondrial DNA has been intensively analysed with biochemical approaches (reviewed in[5,6]), but little is known about mitochondrial activity at the single cell level. To date, studies on mtDNA replication are widely based on molecular biology (2D-Gel of replication intermediates and *in vitro* assays). Currently available tools, including recent improvements[7], cannot identify mitochondria engaged in DNA replication, and they cannot discriminate the transcription profiles of organelles in single cells. If a technique allowing to detect one mitochondrial transcript at a time has been disclosed[30], it required a genetically engineered step and was shown for the transcript ND6 only.

[0010] Moreover, although sequential RNA and DNA labelling [8], as well as labelling of either RNA or DNA, and proteins[9,10] have been performed, the techniques used, namely immunofluorescence and Fluorescent In Situ Hybridization (FISH), did not permit to simultaneously detect proteins and mitochondrial DNA and RNA. Consequently, available techniques do not render possible a powerful and directly exploitable observation of the course of events occurring during gDNA, especially mtDNA, transcription and replication, especially when different molecular subpopulations, such as DNA, RNA or even proteins, are involved.

[0011] D. JACKSON, 1996 relates to a study investigating the sequences found in attaching loops of nuclear and mitochondrial DNA to underlying structures in human cells. A library of such sequences has been generated. However, all cloned fragments generated in this study bear the property of being transcribed within a cell. This study does not disclose nucleic acid molecules that remain RNA-free during transcription and replication of the nuclear and mitochondrial genome.

[0012] SOLOVEI IRINA et al., 2010 discloses protocols for a 3D-FISH method applied on cultured cells combined with immunostaining.

[0013] ALINE V PROBST ET AL, 20 April 2007 discloses using 3D-FISH for visualizing the spatial organization of the nucleic DNA and to follow the dynamic unfolding of the centromeric regions by staining the nucleic DNA in a cell.

[0014] P.S. MASNY, 30 June 2004 discloses the use of 3D-FISH for studying the nuclear organization of the FSHD genomic region.

[0015] ZARDOYA ET AL, 1 April 1996 discusses the determination of the complete nucleotide sequence of the African lungfish (*Portopterus dolloi)* mitochondrial genome through cloning said genome into plasmids and subsequent sequencing. ZARDOYA ET AL does not disclose any nucleic acid molecule suitable for use as a probe for detecting the occurrences of initiation of replication events in mitochondrial DNA.

[0016] WO2005/003766 discloses a method of regulating metabolism and mitochondrial functions in a cell, by contacting the cell with particular agents. A list of mitochondrial proteins is provided, with reference to databases access numbers.

[0017] ALAN L ET AL 1 July 2010 discloses an experiment of co-localization on mitochondrial DNA using labelled probes targeting either ND5 mRNA or the complementary mRNA permanently attached to the mitochondrial D-loop. D7 does not disclose a staining with probes targeting mitochondrial DNA,

[0018] XING Y ET AL 26 February 1993 relates to the visualization of fibronectin and neurotensin messenger RNAs within mammalian cells by flurorescence hybridization with genomic complementary DNA and intron-specific probes. The visualization of the spatial repartition of different categories of mRNAs within said cells is sought, distinguishing RNA free to diffuse, or RNA localizing to the nuclear interior, in order to conclude to a nuclear function closely integrated with the cell structure.

[0019] None of these documents deal with the problem of detecting both the occurrence of initiation of replication events in mitochondrial genomic DNA and the mitochondrial DNA transcription in a eukaryotic cell.

[0020] The present invention addresses the need to obtain an outstanding tool for studying DNA replication, allowing a deeper comprehension of the events associated with DNA replication, and including the comprehension of the coordination of these events with other events such as RNA transcription and even protein(s) distribution in cell(s) or tissue(s) by tracking and monitoring these distinct molecular subpopulations in a concomitant or even simultaneous manner. Consequently, the invention proposes a new way to further explore the complex cellular dynamics and to use such exploration in detection of pathological states.

[0021] According to the present invention novel information can be provided on the dynamics of mitochondrial gDNA processing during physiological and pathological processes. These findings have implications in diagnostic tools of diseases, especially mitochondrial diseases or diseases associated with mitochondrial dysfunction(s) or impairment, in particular those where mtDNA depletion and mtDNA loss can be observed.

[0022] Indeed, defects in the mitochondrial replication machinery can lead to loss of genetic information by deletion and/or depletion of the mitochondrial (mt) DNA, which subsequently may cause disturbed oxidative phosphorylation and neuromuscular symptoms in patients. mtDNA depletion can originate from genetic defects, or be acquired, i.e by clinical treatments, as for prolonged administration of anti-HIV nucleoside analogues. qPCR analysis on mtDNA is currently used to detect alterations of the mtDNA content in a given cell population. By permitting the monitoring of the occurrence of initiation of replication events in mitochondrial genomic DNA, and for example by measuring these events and following their evolution during the progression of disease, the present invention provides for the determination of such an impaired or abolished function. Consequently, detecting the initiation of DNA replication events and optionally combining this detection with the detection of other signals, therefore determining the state of mtDNA at the single cell level, enables the emergence of a more powerful research and diagnostic tool.

[0023] To this end, the invention relates to a method for both the detection of the occurrence of initiation of replication events in genomic DNA, which is mitochondrial genomic DNA, and the detection of mitochondrial DNA transcription in a eukaryotic cell, comprising the steps of:

- contacting said eukaryotic cell comprising mitochondrial genomic DNA with a first nucleotide probe, under conditions enabling *in situ* hybridization of said first nucleotide probe with a target region in the mitochondrial DNA genome, wherein said target region comprises a nucleic acid sequence which has no identified corresponding annealing RNA in a metabolically active cell and therefore remains RNA-free during transcription and replication of said mitochondrial DNA genome, said first nucleotide probe being specific for a segment of non transcribed mitochondrial gDNA and comprises or consists of :
    1. i. the nucleic acid having the sequence of any one of SEQ ID N°1 to SEQ ID N°16 or a complement thereof, or,
    2. ii. a nucleic acid that has at least 80% identity with the nucleic acid sequence of any one of SEQ ID N°1 to SEQ ID N°16 or a complement thereof,

  said nucleic acid molecule being either a single stand molecule or a double strand molecule, and,
- contacting said eukaryotic cell with at least a second nucleotide probe targeting at least one mitochondrial RNA molecule corresponding to a transcribed region of a mitochondrial DNA molecule in the cell, and
- detecting said first nucleotide probe hybridized to said mitochondrial DNA and said second nucleotide probe hybridized to said mitochondrial RNA molecule, as claimed in claim 1.

[0024] Other aspects of the method of the invention are found in claims 2 to 12.

[0025] According to a particular embodiment, the target region or the nucleic acid sequence of the target region that has an ability to remain RNA-free, is located in a naturally transiently open structure of two complementary single strands of gDNA in a metabolically active cell.

[0026] According to a particular embodiment, such a naturally transiently open structure is a replication bubble originating around the locus of a replication origin. According to a particular embodiment, such a naturally transiently open structure is the so-called DNA encompassed by the D-loop region of the mitochondrial genome, which is located between coordinates 16024 to 576 in the human mitochondrial genome (NCBI or Genbank or MITOMAP sequence reference NC_012920.1). According to a particular embodiment, the sequence of the target region that has an ability to remain RNA-free, is located proximal to, or includes, or overlaps known origin(s) of replication, in particular a mitochondrial origin of replication, or is within a distance of less than 10 nucleotides, in particular 1, 2, 3, 4, 5, 6, 7, 8, 9, especially 6 nucleotides, from a known origin of replication, in particular a mitochondrial origin of replication. In particular in human mtDNA, such an origin of replication can be the O_H origin of replication, located between coordinates 110 to 441 of the mitochondrial genome (NCBI or Genbank or MITOMAP sequence reference C_012920.1).

[0027] According to a particular embodiment said target region or said nucleic acid sequence of the target region encompasses nucleotides within a distance of less than 10, in particular 1, 2, 3, 4, 5, 6, 7, 8 or 9 nucleotides upstream or downstream from a naturally transiently open structure of two complementary single strands of gDNA in a metabolically active cell.

[0028] In the context of the invention, "hybridization" relates to the fact of obtaining a close interaction of the nucleotide probe and the target region that is expected to be revealed by the detection of the nucleotide probe. Such an interaction can be achieved by the formation of hydrogen bonds between the nucleotide probe and the target sequence, which is typical of the interactions between complementary nucleotide molecules capable of base pairing. Hydrogen bonds can be found, for example, in the annealing of two complementary strands of DNA.

[0029] The nucleic acid sequence of the probe should be at least partly complementary to the sequence of the target region of the genomic DNA, i.e. should be complementary over a region sufficient to enable stable base pairing.

[0030] Typically, a first nucleotide probe designed for hybridizing to a target region of genomic DNA is a labelled nucleic sequence fragment complementary to the target region of the genomic DNA and having substantially or in particular exactly, the same length as said target.

[0031] In another particular embodiment, the first nucleotide probe designed for hybridizing to a target region of genomic DNA is a labelled nucleic sequence fragment complementary to the targeted DNA fragment and having substantially, or in particular exactly, the same length than the nucleic acid sequence which has no identified corresponding annealing RNA in a metabolically active cell and therefore remains RNA-free during transcription and replication of said DNA genome.

[0032] In a particular embodiment, a first nucleotide probe designed for hybridizing to a target region of genomic DNA is a labelled nucleic sequence fragment comprising a nucleic acid sequence that is complementary to the targeted DNA fragment, said nucleic acid sequence having substantially, or in particular exactly, the same length than the nucleic acid sequence which has no identified corresponding annealing RNA in a metabolically active cell and therefore remains RNA-free during transcription and replication of said DNA genome.

[0033] However, according to other embodiments, the interaction of the nucleotide probe and the target region can also involve van der Waals interactions, ionic bonds or covalent linkages. Such interaction(s) might imply that the nucleotide probe contains modified nucleotides or bear specific moieties generally not present in nucleotidic molecules.

[0034] "*In situ* hybridization" refers to the fact that the hybridization is carried out on the assayed biological material. Said biological material can be single cell(s) or tissue(s), or a sample comprising the same. Preferably, the integrity of the structure and/or content of the biological material is maintained. Therefore, in order to carry out the invention, the biological material is preferably fixed.

[0035] Accordingly, in a preferred embodiment, the method of the invention is carried out on fixed cell(s) or tissue(s).

[0036] In a particular embodiment, the method of the invention further permits to maintain the integrity of the cell(s) volume and thus the analysis of fixed sample(s) in three-dimension.

[0037] According to a particular embodiment, the cell(s) or tissue(s) are eukaryotic cell(s) or tissue(s), in particular human cell(s) or tissue(s). For illustration cell(s) or tissue(s) derived from human cell lines such as HeLa, HCT116, HT29, AGS cell lines, and/or human primary cells, i.e. IMR-90, BJ human fibroblasts obtained from ATCC, are used.

[0038] According to the invention, genomic DNA is mitochondrial gDNA.

[0039] According to another aspect of the present disclosure, the genomic DNA may be nuclear gDNA.

[0040] In another particular aspect, the genomic DNA may be both nuclear gDNA and mitochondrial gDNA. In such a context, the expression "genomic DNA "refers to a group of genomic DNA molecules, i.e. refers to more than one genomic DNA molecule, said group of genomic DNA molecules consisting of more than one copy of genomic DNA molecules (as found in a single mitochondrion) and/or more than one genomic DNA molecules that are different from each other (such as mitochondrial gDNA and nuclear gDNA).

[0041] According to the invention, a "probe" is aimed at revealing the target region of interest, and is therefore generally, but non-exclusively, labelled. Labelling of the probe aimed at revealing the target region in the DNA genome is preferably achieved with either radio- or antibody-discoverable- or fluorescent- or biotinylated- tags or quantum dots, especially fluorescent quantum dots. Said tags or quantum dots are directly or indirectly associated, including coupled, to the probe. Depending upon the type of labelling, the probe can be localized or visualized or measured on the biological material after hybridization with its target using appropriate techniques, such as autoradiography or fluorescence microscopy. An example of discoverable tag is digoxigenin,

biotin, or hapten for example revealed by a labelled antibody or a labelled reagent, such as a fluorescent antibody raised against digoxigenin or a labelled biotin binding molecule such as avidin or streptavidin.

[0042] According to a specific embodiment, the probe is rendered discoverable, especially through fluorescence detection methods, by introducing an antigen in said probe or by coupling said probe with an antigen that will be further revealed by a secondary anti-antigen antibody, especially a fluorescent anti-antigen antibody. One advantage of using antibodies might be an increase of the intensity of the resulting fluorescent signal.

[0043] In a particular embodiment, probe(s) are directly labelled with fluorescent moieties (tags). One advantage of such an embodiment might be to bypass the use of an antibody for the detection of the probe in order, for example, to increase the specificity or the practicability of the labelling/detection method.

[0044] Probe(s) is/are preferably nucleotide probe(s), and are especially short sequences of single stranded DNA capable of base pairing with their complementary DNAs. Probe(s) encompass(es) probe(s) containing nucleotide(s) coupled or linked to other molecule(s) or moiety(ies).

[0045] According to a particular embodiment probe(s) is/are DNA probe(s) such as PCR product(s) or DNA fragment(s), including plasmidic probe(s) or probe(s) comprising such elements.

[0046] According to a particular embodiment, they can be double-stranded DNA probes that require being denaturated as single stands prior to their use.

[0047] The nucleotide probe may contain modified nucleotides or bear specific moieties generally not present in nucleotidic molecules. Locked Nucleic Acids (LNA) are modified nucleotides and a class of
RNA analogs that have an exceptionally high affinity towards complementary DNA and RNA. They can substitute natural nucleotides in DNA probes.

[0048] In the context of the invention, a "target region" in the DNA genome is a genomic DNA region comprising a nucleic acid sequence which has no identified corresponding annealing RNA in the metabolically active cell under assay and therefore remains RNA-free during transcription and replication of the DNA genome to which the nucleic acid sequence belongs.

[0049] In other words, a target region is a genomic DNA region that has ability to remain RNA free, in particular RNA-transcript(s) free, during the transcription and replication of the DNA genome to which it belongs (mtDNA or nDNA) in the cell that is tested.

[0050] According to a specific embodiment, the target region consists of a nucleic acid region in a genomic DNA which has no identified corresponding annealing RNA in a metabolically active cell.

[0051] By "occurrence" it is meant that the method of the invention enables to qualitatively detect initiation of the replication of genomic DNA and, according to a particular embodiment, to quantitatively detect such initiation event(s) of the replication process.

[0052] "Initiation of replication events" can be, for example, the formation of replication bubble(s) on the analyzed genomic DNA, or in a particular embodiment where the mitochondrial gDNA is assayed for initiation of replication, the formation of a D-loop structure, including the formation of three-stranded D-loop structure. Such events may precede the entire replication of the analyzed gDNA, meaning that such events may precede replication over the complete analyzed gDNA. Such events may alternatively be followed by interrupted synthesis of the nascent strand of DNA.

[0053] In a cell, DNA replication usually begins at specific location(s) in the genome, called "origin(s)" or "replication origin(s)". Once polymerases have opened the double stranded genomic DNA molecule, an area known as a "replication bubble" forms (usually initiated at a certain set of nucleotides, the origin of replication).

[0054] With respect to particular embodiments aimed at detecting the occurrence of initiation of replication events in mitochondrial genomic DNA, it is knowledgeable to consider that, in Mammalian, Avian, Fish, or Plant cells, the initiation of mitochondrial genome replication generally occurs in a particular region named "control region" or "D-Loop" or "displacement loop". When the mtDNA initiation of replication starts, the D-Loop region is opened, and the corresponding DNA locally results in single strands that serve as template for the synthesis of new mtDNA. This transitory opened D-Loop region may present a triplex-DNA structure, said structure being however located in a naturally transiently open structure of two complementary single

strands of gDNA. The presence of D-loop region is typical of the human and other mt DNAs, such as Mammalian, Avian, Fish or Plant mtDNAs. The coordinates of said D-loop regions vary according to the considered organisms but can be found in the literature[31]. However D-Loop regions are not found in all mtDNA.

[0055] In particular in human cells, the D-loop region is roughly located between the coordinates 16024 and 576 of the L-strand on the mitochondrial genome (according to the data released to date on databases, in particular under accession number NC_012920.1 (NCBI, GenBank or MITOMAP sequence reference), see in particular MITOMAP: http://www.mitomap.org/MITOMAP/HumanMitoSeq).

[0056] In a particular embodiment, the target region or the RNA-free nucleic acid sequence comprised in said target region is located in a naturally transiently open structure of two complementary single strands of gDNA in a metabolically active cell, as disclosed above and in the following embodiments.

[0057] According to a particular embodiment, the target region or the RNA-free nucleic acid sequence comprised in said target region is located upstream from the major H-strand promoter on the mitochondrial genome (PH1), which coordinates are given in Table 2 (coordinates and direction are given herein with respect to the L-strand of the mtDNA). However, in the context of the present disclosure, the target region or the RNA-free nucleic acid sequence comprised in said target region encompasses the sequences found on either the L-strand or the H strand at the specific location mentioned herein. Reference is made to the L-strand to indicate the position of the major H-strand promoter only.

[0058] According to a particular embodiment, especially when probe(s) are synthesized or obtained as a result of a PCR amplification, probe(s) is/are double-stranded DNA probe(s). They are denaturated as single stands prior to their use. When used simultaneously after denaturation, such a mix of complementary single-stranded probe(s) results in annealing both the L and H strands of the target region of a mtDNA.

[0059] In a particular embodiment, the target region or the RNA-free nucleic acid sequence comprised in said target region is located downstream from the L-strand promoter on the mitochondrial genome (LP or LSP), which coordinates are given in Table 2 (coordinates and direction are given herein with respect to the L-strand of the mtDNA). However, in the context of the present disclosure, the target region or the RNA-free nucleic acid sequence comprised in said target region encompasses the sequences found on either the L-strand or the H strand at the specific location mentioned herein. Reference is made to the L-strand to indicate the position of the L-strand promoter only.

[0060] By "naturally transiently open structure of two complementary single strands of gDNA" it is meant a gDNA structure formed by the dissociation of the two DNA strands constituting the gDNA as a result of processing by replication machinery and mechanism(s) inherent to a metabolically active cell, during its life cycle.

[0061] More specifically, it will be understood that the target region is located near or in a region of the genomic DNA that is involved in the early events of the replication of said genomic DNA, such as a region found in a replication bubble or a region at least partly encompassed by a replication bubble. Such a region will generally be localized in the vicinity of a replication origin of a genomic DNA of an eukaryotic cell, in particular in the close vicinity or near, i.e. no farther than 10 nucleotides from a replication origin of a genomic DNA of an eukaryotic cell.

[0062] According to a particular embodiment, the target region is located in the vicinity of a replication bubble or at a locus encompassed by a replication bubble (where a replication bubble can be found), in particular no farther than 10 nucleotides from such a bubble or locus encompassed by such a bubble. Replication bubbles initiate at the locus of replication origins.

[0063] According to a specific embodiment, the target region is located at 5 nucleotides of the O_H replication origin in the human mtDNA.

[0064] A nucleic acid sequence having "no identified corresponding annealing RNA in a metabolically active cell" is a sequence having no strictly corresponding, i.e. complementary or matching, RNA, especially no RNA transcript(s) resulting from the transcription process occurring naturally in the living eukaryotic cell under assay. By "corresponding" it is understood a substantial, in particular a strict, complementarity of nucleic acid sequences which are aligned and whose similarity is calculated over the entire length of the aligned sequence by alignment algorithm such as the Needelman and Wunsch algorithm (a substantial similarity or perfect match is expected). Therefore such a nucleic acid sequence has an ability to remain RNA-free, in particular RNA-transcript(s) free, within the analyzed cell, meaning that such a nucleic acid sequence will not give rise to any identified RNA molecule, especially a RNA molecule that would have been transcribed from genomic DNA in the analyzed cell, nor

hybridize with RNA primers involved in replication process in a metabolically active cell containing said nucleic acid sequence. Such a nucleic acid sequence cannot be detected by a probe aimed at detecting the result of transcription events occurring in a cell.

[0065] It is pointed out that such a sequence may be identified starting from the literature describing transcription and replication processes of gDNA or in databases, having regard to annotation(s) available in said databases, or as a result of deductions arising from said annotations.

[0066] Consequently, a first nucleotide probe whose sequence would strictly match the sequence of a nucleic acid sequence as discussed above, would not hybridize with any RNA molecule naturally expressed within a metabolically active cell.

[0067] Such a nucleic acid sequence is thus characterized in that it does not bear any coding information that would be reflected at the transcription level of the DNA processing in a cell.

[0068] By "conditions enabling in situ hybridization" it is meant that the target region is rendered physically accessible to the probe in order to enable the hybridization of said probe to the target region.

[0069] According to a particular embodiment, the hybridization of the probe to the target region may be only partial along the entire length of the probe or the target region, but sufficient to be specific and stable during washing step(s) following the hybridization.

[0070] According to another embodiment, the hybridization of the first nucleotide probe to the target region occurs over the length of the probe and/or over the length of the target region.

[0071] According to a particular embodiment, to render the target region accessible to the probe in order to enable the hybridization of said probe to the target region, said target region has to be available under the form of an accessible single stranded of gDNA even transiently during the replication process. According to a particular embodiment, it is the nucleic acid sequence which has no identified corresponding annealing RNA that has to be available under the form of an accessible single stranded of gDNA even transiently during the replication process.

[0072] According to a particular embodiment, the first nucleotide probe strictly anneals to the above-mentioned nucleic acid sequence comprised in the target region that has no identified corresponding annealing RNA in a metabolically active cell. In other words, in said embodiment, when hybridizing to the nucleic acid sequence comprised in the target region which has no identified corresponding annealing RNA, the first probe does not overflow the boundaries of said nucleic acid sequence.

[0073] Considering the mitochondrial genomic DNA, the D-loop region of said DNA can be found as a specific structure involving a three-stranded DNA structure that is formed when a newly synthesized single DNA strand remains bound to one of the parental DNA strand of the gDNA and displaces one of the duplex parental strand.

[0074] When present, such a three-stranded DNA structure might help rendering a target region located in the D-loop structure or in the vicinity of this structure accessible to the probe. The target region is, in this configuration, either located in a naturally transiently open segment of two complementary single strands of gDNA when the mitochondrial genomic DNA is entering replication, or in a region which is impacted by the presence of the third DNA strand that might help to push aside proteins or other elements that might render the target region crowded and/or hinder the target region with the result of rendering said region inaccessible to the probe for subsequent hybridization of said probe to the target region.

[0075] In the context of the invention however, initiation of replication is considered an event to be detected in mitochondrial gDNA even when the presence of a third DNA strand in a D-loop does not further give rise to the replication of the whole mitochondrial DNA strand. In other words, the initiation of the replication of the mitochondrial gDNA is considered to happen with the formation of a replication bubble, including the formation of a D-loop around the locus of a replication origin.

[0076] Since the method of the invention is applied to the detection of the occurrence of initiation of replication events in mitochondrial genomic DNA, the method of the invention can permit the specific detection of the D-loop region opening by labeling the mitochondrial genomic DNA with a probe as defined in the claims hybridizing at least partly the target region located in the vicinity of said D-loop or at a locus included in said D-loop.

[0077] According to a particular embodiment, the first nucleotide probe strictly anneals to the above-mentioned nucleic acid sequence comprised in the target region that has no identified corresponding annealing RNA in a metabolically active cell.

[0078] According to a particular embodiment, the accessibility of the target region to the first probe as defined in the claims can be improved by performing a step aimed at partially denaturing the genomic DNA molecule comprising the target region, for example by heating the eukaryotic cell comprising said genomic DNA at a temperature in the range of 72 to 78°C, preferably 75°C, for 2 to 8 minutes, preferably 4 to 5 minutes, in particular 5 minutes, prior to the hybridization step.

[0079] According to a particular embodiment, said partial denaturation is performed without using any chemical agent resulting in a complete denaturation of nucleic acids. Consequently, treatments with HCl or Pepsin, alkaline agents or ethanol are prohibited. Conversely, the use of chemical agents and/or temperature conditions enabling or assisting a partial denaturation of nucleic acids is possible. An example of chemical agent that can be used is formamide. Combinations between the proposed treatments disclosed herein are encompassed by the present invention. By "partial denaturation" it is meant that the two strands constituting a double stranded nucleic acid are not found completely separated i.e. under the form of single strands, after such a denaturation. According to a specific embodiment, said partial denaturation results in increasing the size of opening(s) or bubble(s) that could be found on the double-stranded nucleic acid of gDNA prior to eliciting its partial denaturation.

[0080] According to a particular embodiment wherein temperature and/or chemical agent(s) is(are) used to assist the partial denaturation of the double-stranded target nucleic acid, said agent(s) enable(s) the partial denaturation by performing or assisting the increase in size of opening(s) or bubble(s) on the double-stranded target nucleic acid, to the exclusion of the result consisting in the dissociation of the strands of the double-stranded target nucleic acid, on their whole length.

[0081] In the context of the invention, both the initiation of genomic DNA replication in a cell or tissue and produced RNA molecules, being for example RNA molecules corresponding to transcription products of genomic DNA fragments concomitantly transcribed in said cell or tissue, are tracked.

[0082] Thus, the method of the invention is used for the further detection of at least one RNA molecule corresponding to a transcribed region of a DNA molecule in an eukaryotic cell, which comprises the step of contacting said eukaryotic cell expressing said RNA molecule with at least a second nucleotide probe, and detecting said second nucleotide probe after hybridization with said RNA molecule.

[0083] According to a particular embodiment, the DNA molecule giving rise to the RNA transcript molecule detected by the second nucleotide probe is a genomic DNA inside said cell (the analyzed cell).

[0084] In a particular embodiment, the labelling of the nucleic acid sequence of the target region on the DNA genome and the RNA molecule is achieved in one step, in particular simultaneously.

[0085] In a particular embodiment, the detection of the nucleic acid sequence of the target region on the DNA genome and the RNA molecule is achieved in one step, in particular simultaneously.

[0086] In the context of the invention, the hybridization of the second nucleotide probe to a RNA molecule corresponding to a transcribed region of a DNA molecule is achieved by obtaining a close interaction of the nucleotide probe and the RNA molecule that is expected to be revealed by the detection of the nucleotide probe. Such an interaction can be achieved by the formation of hydrogen bonds between the nucleotide probe and RNA molecule, which is a typical example of the interactions between complementary nucleotide molecules. Hydrogen bonds can be found, for example, in the annealing of two complementary strands of DNA.

[0087] Typically, a nucleotide probe designed for hybridizing to a RNA fragment (RNA molecule) is a labelled nucleic sequence fragment complementary to the RNA fragment to detect. The nucleic acid sequence of the probe should be at least partly complementary to at least a part of the RNA molecule to detect, i.e. should be complementary over a region sufficient to enable stable base pairing.

[0088] However, according to other embodiments, the interaction of the nucleotide probe and the RNA molecule can also involve van der Waals interactions, ionic bonds or covalent linkages. Such interaction(s) might imply that the nucleotide probe contains modified nucleotides or bear specific moieties generally not present in nucleotidic molecules.

[0089] In a particular embodiment, probe(s) are directly labelled with fluorescent moieties (tags).

[0090] Probe(s) is/are preferably nucleotide probe(s), and are especially short sequences of DNA or RNA (cRNA probes or

riboprobes) that binds to their complementary RNAs. Probe(s) encompass(es) probe(s) containing nucleotide(s) coupled or linked to other molecule(s) or moiety(ies).

[0091] The nucleotide probe may contain modified nucleotides or bears specific moieties generally not present in nucleotidic molecules. Locked Nucleic Acids (LNA) are modified nucleotides and a class of RNA analogs that have an exceptionally high affinity towards complementary DNA and RNA. They can substitute natural nucleotides in DNA or RNA probes.

[0092] The invention encompasses the use of probes suitable for revealing several distinct RNA molecules or fragments thereof, or the use of a single probe targeting distinct RNA molecules or fragments thereof (specific of a pool of RNA molecules or fragments thereof), or the use of a single probe specific of the sequence of a unique RNA molecule or fragment thereof within a cell or tissue. In this context the term "probe" encompasses a plurality of molecular entities used together to reveal one or many RNA molecules, said RNA molecules being distinct or different.

[0093] According to the invention, such a probe suitable for revealing RNA molecule(s) is generally, but non-exclusively, labelled. Labelling of the probe can be achieved with either radio- or antibody-discoverable- or fluorescent- or biotinylated- tags or quantum dots, especially fluorescent quantum dots. Said tags or quantum dots are directly or indirectly associated, including coupled, to the probe. According to the type of labelling, the probe can be localized in the biological material using appropriate techniques, such as autoradiography or fluorescence microscopy, respectively. The developments made above with respect to the labelling of the probe aimed at revealing the target region in the DNA genome are also applicable to the labelling of the probe(s) aimed at detecting RNA molecule(s).

[0094] Detected RNA molecule(s) can be polycistronic RNA, RNA corresponding to transcribed fragments of genomic DNA (nuclear and/or mitochondrial genomic DNA), processed or unprocessed RNA(s) in said cell.

[0095] In a particular embodiment of the invention, the first probe aimed at revealing a gDNA target region is a single stranded DNA fragment ranging in size from 80 bp to 3000 bp, or from 90 to 150 bp, in particular from 95 to 110 bp, preferably sizing 99 bp.

[0096] Considering the second probe aimed at revealing RNA molecule(s), according to a particular embodiment, said second probe is a single stranded nucleotidic DNA fragment ranging in size from 100 bp to 3000 bp, and preferably sizing between 100bp and 2000bp when aimed at detecting mitochondrial transcripts.

[0097] According to a particular embodiment, the size and/or sequence of second probe(s) aimed at revealing RNA molecule(s) is particularly adapted to enable the detection of transcription products resulting from the transcription of coding segments of genomic DNA, especially segments corresponding to genes.

[0098] As stated above, the nucleic acid molecule suitable for use as first nucleotide probe is specific for a segment of a non transcribed mitochondrial gDNA, especially an entirely non-transcribed mitochondrial gDNA, according to the definition provided herein.

[0099] Accordingly, the first nucleotide probe or molecule is complementary of a genomic DNA region that has no corresponding RNA transcript at a cellular level, and therefore remains RNA-free at the cellular level.

[0100] In a particular embodiment, the first nucleotide probe or molecule targets the genomic DNA sequence localized between the two promoters *PH1* (or *HSP*-Heavy Strand Promoter) and *LSP* (Light Strand Promoter) of the mitochondrial genome of a eukaryotic cell. Both the *HSP* and *LSP* promoters are found in all eukaryotic mtDNA although their name might differ depending on the species to which the considered eukaryotic mtDNA belongs. The corresponding names can be identified through the literature.

[0101] The respective position (coordinates) of these two promoters on the human mitochondrial genome (NCBI, GenBank or MITOMAP sequence reference C_012920.1) is indicated in Table 2.

[0102] In the human mitochondrial genome, such a first probe or nucleic acid molecule sequence has been designated mREP by the inventors. Coordinates of this sequence are given in Table 1 (NCBI, GenBank or MITOMAP sequence NC_012920.1, used as reference). mREP (SEQ ID No 1) is also disclosed in Fig. 13a.

[0103] In another embodiment, said first probe comprises the mREP sequence (SEQ ID No 1).

[0104] In a specific embodiment, the first nucleotide probe or molecule is complementary of a nucleic acid sequence that has at least 80% identity with the target DNA region that is localized between the two promoters *PH1* (or *HSP)* and *LSP* of the mitochondrial genome of a eukaryotic cell, or has at least 80 % identity with mREP.

[0105] By "at least 80 % homology" it is meant that their sequence of nucleotides differ from less than 20%, calculated over the entire length of the considered sequence (global alignment calculated for example by the Needleman and Wunsch algorithm).

[0106] The genomic DNA sequence localized between the two promoters *PH1* (or *HSP)* and *LSP* of the mitochondrial genome of a eukaryotic cell is part of a highly variable region in the mitochondrial genome. Fig 13b discloses polymorphism variations known to date with respect to the human mREP sequence (SEQ ID No 1) part of the invention. However, very few of these variations can be found simultaneously in an individual, i.e. only 1, 2, 3 or 4 of these variations can be found simultaneously. Said variations are generally linked to a subpopulation type (e.g. Caucasian, African...). These polymorphism variations are a basis for the design of variants of mREP.

[0107] Table 3 discloses the percentages of identity between the sequences corresponding to the mREP probe (SEQ ID No 1) in several organisms and species, with respect to human mREP probe, along with coordinates of said sequences on the corresponding mitochondrial genomes (SEQ ID No 2 to 16).

Table 3 Percentages of identity between the sequences (SEQ ID No 2 to 16) corresponding to the mREP (SEQ ID No 1) probe in several organisms and species. Refseq are NCBI or GenBank reference numbers.

Organism	Accession number Refseq	mtDNA Genome size (bp)	mREP alignment coordinates
Primates			
Homo sapiens	NC_012920.1	16 569	446-544
Pan troglodytes	NC_001643.1	16 554	16424-16521
Pan paniscus	NC_001644.1	16 563	16433-16530
Gorilla gorilla	NC_001645.1	16 364	16233-16332
Pongo pygmaeus	NC_001646.1	16 389	16247-16357
Hybolates lar	NC_002082.1	16 472	16148-16246
Cebus albifrons	NC_002763.1	16 554	15946-16044
Other mammalians			
Capra hircus	NC_005044.2	16 643	15519-15630
Mus musculus	NC_005089.1	16 299	15654-15757
Oryctolagus cuniculus	NC_001913.1	17 245	15767-15867
Canis lupus	NC_008092.1	16 729	16456-16555
Rattus sordidus	NC_014871.1	16 309	15643-15744
Felis catus	NC_001700.1	17 009	759-864
Castor canadensis	NC_015108.1	16 701	15766-15866
Avian			
Gallus gallus	NC_001323.1	16 775	473-571
Fish			
Danio rerio	NC_002333.2	16 596	682-782

[0108] In a specific aspect of the present disclosure, the first nucleotide probe comprises a nucleic acid molecule having the sequence of any one of SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5, SEQ ID N°6, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12, SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°16, or the sequence that is complementary of any one of SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5, SEQ ID N°6, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12, SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°16, or is a fragment of said sequences.

[0109] In a specific aspect of the present disclosure, the first nucleotide probe comprises a nucleic acid molecule encompassing fragments of the nucleic acid sequence of any one of SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5, SEQ ID N°6, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12, SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°16, or the nucleic acid sequence that is complementary of any one of SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5, SEQ ID N°6, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12, SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°16.

[0110] In a specific embodiment of the invention, the first nucleotide probe consists of a nucleic acid molecule that has the nucleic acid sequence of any one of SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5, SEQ ID N°6, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12, SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°16, or the nucleic acid sequence that is complementary of any one of SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5, SEQ ID N°6, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12, SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°16.

[0111] According to a particular embodiment of the invention, the first nucleotide probe is a nucleic acid that has at least 80% identity with the nucleic acid sequence of any one of SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5, SEQ ID N°6, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12, SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°16, or the nucleic acid sequence that is complementary of any one of SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5, SEQ ID N°6, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12, SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°16.

[0112] The first nucleotide probe may encompass a mix of more than one, but distinct probes, especially probes comprising nucleic acid molecules having distinct sequences from each other, chosen among the sequences disclosed above. The first nucleotide probe may encompass a mix of two nucleic acid molecules having sequences that are complementary to each other. Such a mix might have to be denaturated prior to its use. Fig 13b discloses polymorphism variations known to date with respect to the mREP sequence. These polymorphism variations may be used to design probes alternative to mREP corresponding to SEQ ID No 1.

[0113] Fig 13c discloses alignments between the human mREP sequence and the corresponding sequences in different organisms, which are also disclosed herein under SED ID No 2 to 16.

[0114] In a particular embodiment, the first nucleotide probe is directly labelled, in particular with a fluorescent group.

[0115] Labelling can be achieved with groups or labels such as Biotin, digoxin and digoxigenin (DIG), alkaline phosphatase and fluorescent groups or labels such as fluorescein (FITC), Texas Red and rhodamine or derivatives thereof, or dyes including coumarins, rhodamines, carbopyronins, oxazines or derivatives thereof or quantum dots, especially fluorescent quantum dots, or derivatives thereof.

[0116] By "directly labelled", it is meant that the detection of the label does not require the intervention of another, i.e. secondary, chemical agent or compound, including an antibody, to be achieved. Such a direct labelling might improve the specificity of the labelling.

[0117] Labelling can be achieved through a commercial kit, for example according to a nick translation procedure. An example of such a commercial kit is the Nick Translation Atto NT Labeling kit from JenaBioscience; comprising the following dyes: Atto425=blue, Atto488=green, Atto550=red.

[0118] In a particular embodiment, the first nucleotide probe comprises modified nucleotides, as disclosed herein.

[0119] The invention also relates to a method for the *in situ* hybridization and detection of mitochondrial nucleotidic material within at least one eukaryotic cell, which comprises the steps of:

1. a. Fixation of said cell in 1 to 4% paraformaldehyde (PFA), preferably 2% PFA for about 20 to 30 minutes, especially 30 minutes,
2. b. Permeabilization of said fixed cell with 0.5% to 1% Triton X100 in PBS (Phosphate Buffered Saline Buffer) 1X, for about 5 to 10 minutes at 4°C, especially 5 minutes,
3. c. Denaturation of the nucleic acid contents of said permeabilized fixed cell by heating at a temperature in a range of 72 to 78 °C, preferably 75°C, for 2 to 8 minutes, preferably 4 to 5 minutes, especially 5 minutes,
4. d. Contacting the nucleic acid(s) in the cell treated according to step (c) with mitochondrial nucleotide probe(s) to enable

hybridization of mitochondrial nucleic acid(s) with said probe(s), wherein the probe(s) has(have) a size ranging from 80 to 3000 nucleotides, or from 90 to 1000 nucleotides, in particular from 95 to 110 nucleotides, said nucleotide probe(s) being contained in an hybridization solution comprising from 100ng/µl to 10µg/µl of salmon sperm DNA,

5. e. Detecting the nucleic acid(s) hybridized to the probe(s) added in step (d).

[0120] The denaturation step of said method can further be carried out in an appropriate buffer and/or with a chemical agent aimed at partially denaturing nucleic acids, as disclosed herein.

[0121] According to a specific embodiment of the invention, the denaturation step of the nucleic acid contents of a fixed cell is carried out by heating at a temperature in a range of 72 to 78 °C, preferably 75°C, for 2 to 8 minutes, preferably 4 to 5 minutes, especially 5 minutes, in the presence of a chemical agent such as formamide, especially a solution of 70% formamide or 70% formamide/2xSSC.

[0122] Said method can further comprise a step of washing the cell(s) contacted with the nucleotide probe(s) with an appropriate buffer prior to the detection step.

[0123] According to a particular embodiment, the hybridization step is carried out during about 15 hours at 37°C.

[0124] According to a particular embodiment, the method of the invention further comprises steps enabling labelling and detection of at least one protein of interest within the eukaryotic cell.

[0125] To this end, the cell(s) or tissue(s) under assay can be contacted with antibodies specific for the protein(s) of which detection is sought.

[0126] According to a particular embodiment, the detection of nucleotidic material and of the protein of interest is achieved in one step, in particular simultaneously.

[0127] A step of analysis of the result(s) of the detection(s) might be subsequently performed.

[0128] Fixation of cells can alternatively be performed with agents such as paraffin, acetone, methanol, ethanol, a combination of methanol and acetone a combination of methanol and ethanol, formalin, a combination of paraformaldehyde and methanol, or any combination(s) of the agents disclosed herein.

[0129] Said steps that can be involved in a method as disclosed herein are detailed hereafter.

1. <u>Cell(s) or tissue(s) fixation</u>

[0130] According to a particular embodiment, the fixation of cell(s) or tissue(s) is performed on glass slide(s) with a solution of 1 to 4% paraformaldehyde (PFA), preferably 2% PFA for about 20 to 30 minutes, especially 30 minutes. According to a specific embodiment, the fixation is carried out with a solution of 2% PFA for about 30 minutes. Said fixation can be carried out at room temperature, RT.

[0131] After the fixation, storage of the fixed material can be performed in a buffer such as PBS 1X (during maximum one year at 4°C).

[0132] Alternatively, fixation can be carried out according to any one of the following protocols:

- Acetone Fixation (Fix cells in -20°C acetone for 5-10 minutes.);
- Methanol Fixation (Fix cells in -20°C methanol for 5-10 minutes.);
- Ethanol Fixation (Fix cells in cooled 95% ethanol, 5% glacial acetic acid for 5-10 minutes.);
- Methanol-Acetone Fixation (Fix in cooled methanol, 10 minutes at -20 °C.;Remove excess methanol.);
- Methanol-Acetone Mix Fixation (1:1 methanol and acetone mixture. ; Make the mixture fresh and fix cells at -20 C for 5-10 minutes.);
- Methanol-Ethanol Mix Fixation (1:1 methanol and ethanol mixture, Make the mixture fresh and fix cells at -20 C for 5-10 minutes.);

- Formalin Fixation (Fix cells in 10% neutral buffered formalin for 5-10 minutes);
- Paraformaldehyde-Methanol Fixation (Fix in 4% paraformaldehyde for 10-20 minutes, Rinse briefly with PBS, Permeabilize with cooled methanol for 5-10 minutes at -20 °C.

[0133] While cell(s) or tissue(s) fixation is generally not performed at this stage in standard DNA FISH procedures, standard RNA FISH procedures require the fixation of cell(s) or tissue(s) with 4% PFA for 10 minutes minimum.

2. Cell(s) or tissue(s) permeabilization

[0134] In particular embodiments, the permeabilization step might be required to allow a good infiltration of the probes, especially through cell membranes, in order for the probes to reach their target sequence. Known chemical reagents used for permeabilizing cells in the prior art are HCl, detergents such as Triton or SDS or Proteinase K.

[0135] According to a particular embodiment, fixed cell(s) or tissue(s) are permeabilized using a 0.5 % to 1% Triton X100 solution in an appropriate buffer, such as PBS 1X, for example during 5 min. Said permeabilization can be carried out at 4°C.

[0136] According to a particular embodiment, fixed cell(s) or tissue(s) can be washed up to 3 times in PBS 1X prior to the permeabilization, and up to 4 times in PBS 1X after the permeabilization.

[0137] According to a particular embodiment, the permeabilization can be followed by incubation of the cell(s) or tissue(s) in 50% Formamide/2X SSC (saline-sodium citrate buffer) in PBS 1X at RT during 30 min, and further switching to 70% Formamide/2X SSC just before the denaturation.

[0138] Optionally, a control assay for the probe specificity can be performed by incubating the samples during 1 hour at 37°C in RNase or DNase solution (100 ug/ml), with an additional washing up to 3 times in PBS 1X prior to the incubation of the cell(s) or tissue(s) in Formamide.

[0139] Standard DNA FISH procedures generally involve a heat treatment of the glass slides (for example during 90°C 1h30 or at 37°C overnight) to remove all enzymes that could interfere with the experiment, and the permeabilization is achieved by incubation with 0.005% Pepsin/0.001 M HCl at 37°C for 15 min, washing in (4%) paraformaldehyde/PBS, then incubating in ethanol series: 70%, 90%, 100%, further followed by RNase treatment that is performed to remove primary transcripts and subsequent washing and dehydratation in ethanol series. The samples are finally air dried.

[0140] Standard RNA FISH procedures generally require permeabilization with Triton X100 0.5% in buffer (the composition of which might contain PIPES, MgCl2, sucrose or NaCl...), washing in 4% PFA/PBS for 10 min on ice and further washings (twice) in 70% ethanol and subsequent dehydratation in ethanol series: 80%, 95%, 100% ethanol and air drying of the samples on a heating plate at 42°C.

[0141] To the contrary, the method of the invention in conducted in absence of enzymatic (i.e. pepsin), acidic (i.e. HCl) or alkaline treatment, alcoholic treatment (i.e. ethanol) or drying agent or treatment (i.e. air dry).

3. Denaturation

[0142] The denaturation step is performed by heating the samples comprising the assayed cell(s) or tissue(s) at a temperature in a range of 72 to 78 °C, preferably 75°C, for 2 to 8 minutes, preferably during 4 to 5 minutes, especially 5 minutes. According to a specific embodiment, the samples are heated at 75°C during 5 minutes. The samples can then be kept on ice until the probe(s) are ready.

[0143] While the denaturation step is generally not performed in standard RNA FISH procedures, because such a step would be unnecessary since RNAs are single stranded molecules, standard DNA FISH procedures generally involve heating the samples at temperatures close to 80°C, and the assistance of other treatments such as chemical or physical treatments usually used to denaturate nucleic acids (e.g. washing with 70% ethanol on ice or dehydratation with ethanol series) in order to obtain single stranded DNA molecules.

[0144] However, the denaturation step according to the invention may be carried out in the presence of chemical agent(s) aimed at partially denaturing nucleic acids, or in an appropriate buffer, such as formamide.

4. Probe design and preparation

[0145] According to the present disclosure, probe(s) are preferably small in size (3 kb or shorter, preferably 1kb or shorter) and the direct use of fluorescence probe(s) for labelling DNA(s) and RNA(s) is preferred.

[0146] When a step of nick translation (direct fluorescence) and Biotin/Digoxigenin (undirect fluorescence) is necessary for probe labelling, thus further requiring primary and secondary fluorescent antibodies to reveal Biotin/Digoxigenin- DNA or RNA probe hybridization, a kit such as the Jena Nick Translation kit can be used to label the probe(s) (Atto fluorescence) and the quantity of DNA recovered after purification should be estimated (about 10% loss compared to input).

5. Pre-hybridisation

[0147] According to a particular embodiment, a pre-hybridization step can be performed, comprising the following steps:

- For 1 slide, mix in 25 µL final volume:
  - 40 ng fluorescent probe (final concentration: 1.6 ng/µl)
  - 400 ng salmon sperm DNA (from 100 ng/µl solution)
  - Buffer: 10% dextran sulfate/50% Formamide/2X SSC in PBS 1X
- Incubate the mix 10 min at 80°C in the dark

[0148] Optionally, pre-hybridized slides can be put to pre cool 30 min at 37°C in the dark before incubating slides.

[0149] Standard DNA and RNA FISH procedures generally require a higher concentration of fluorescent or tagged probe (final concentration of about 10 ng/µl) and the addition of Cot-1 DNA and Salmon Sperm DNA at a final concentration of more than 0.5µg/µL. The mix is then incubated (5 to 10 min) at 74-85°C in the dark.

[0150] According to a particular embodiment, the pre-hybridisation and/or hybridisation step is carried out in absence of Cot-1 DNA.

6. Hybridization

[0151] According to a particular embodiment of the invention, the hybridization step is carried out during about 15 hours at 37°C.

[0152] More specifically, the hybridization step can be done by incubating the samples about 15 hours at 37°C on a heating metal block in the dark. Standard DNA and RNA FISH procedures generally require overnight hybridization.

7. Washes and mounting

[0153] According to a particular embodiment, the method of the invention further comprises a step of washing the cell contacted with the nucleotide probe(s) with an appropriate buffer prior to the detection step.

[0154] For example, washing can be performed up to 2 times for 2 min in 3 mL 2X SSC at RT (cover with black top to protect from light), then up to 2 times in 1X SSC at RT, then up to 2 times in 0.1X SSC at RT. Finally, an ultimate washing can be performed up to 2 times in PBS 1X at RT (all in the dark).

[0155] At this point a simple 2D imaging can be performed. In order to obtain a greater resolution, 3D analysis can be preferred.

8. 3D-FISH

[0156] When 3D-FISH is performed, the following steps may be carried out.

- Incubate 1 h in Hoechst 33342 10 ug/mL final
- Wash 5 times in PBS 1X
- Mount on 20 uL PBS 1X on 70% Ethanol cleaned and dried slides.
- Keep the slides in Dark clean box at RT until confocal analysis.

[0157] As described until this point, the procedure allows the labelling of genomic DNA, especially mtDNA and RNAs. Further steps are required for additional labelling of mitochondrial or cellular proteins.

[0158] According to an advantageous embodiment, the method of the invention further comprises steps enabling labelling and detection of at least one protein of interest within the eukaryotic cell treated for *in situ* hybridization and detection of nucleotidic material. For this purpose, immunofluorescence can be used and the cell can be contacted with antibodies specific for the protein(s) of which detection is sought. Antibodies raised against a particular protein or epitope can be obtained according to usual techniques commonly used in the immunological field.

[0159] In a particular embodiment, the method of the invention enables to achieve the detection of the nucleotidic material and the protein of interest in one step, in particular simultaneously.

9. 3D-FISH coupled Immunofluorescence:

[0160]

The following steps might be performed:

- Incubate the slide with BSA 5% in PBS 1 X 1 h RT in the dark.
- Wash 2 times in PBS 1 X
- Incubate with Primary antibody in BSA 1%, PBS 1X, 1 h RT dark
- Wash 3 times in PBS 1X
- Incubate with Secondary antibody in Hoechst 33342 10 µg/ml final, BSA 1 %, PBS 1X, 1 h RT dark
- Wash 5 times in PBS 1X
- Mount on 20 µL PBS 1X on 70% EtOH cleaned and dried slides.
- Keep the slides in dark clean box at RT until confocal analysis.

Example of a protocol detailing particular steps of a particular embodiment of the method of the invention

[0161] The following procedure is suitable for the detection of the occurrence of initiation of replication events in the mitochondrial genomic DNA of human cell lines and human primary fibroblasts as well as in cells of other eukaryote organisms.

[0162] The design of mtDNA probe(s) used for tracking the occurrence of initiation of replication events has to be adapted to the sequence of the corresponding mitochondrial genomes (see Table 3 that gives correspondence of the mREP probe in other organisms).

1. Cell fixation

[0163]

- Cells Fixation on glass slides: 2% paraformaldhehyde or PFA for 30 min at room temperature, RT.
- Storage in PBS 1X (maximum one year at 4°C)

2. Cell permeabilization

[0164]

- Wash 3 times in PBS 1X
- Permeabilize 5 min at 4°C in 0.5 % Triton X100 in PBS 1X
- Wash 4 times in PBS 1X
- Optional (control assay for the probe specificity) Incubate 1 h at 37°C in RNase or DNase solution (100 ug/ml) Wash 3x in PBS 1 X
- Incubate the cells in 50% Formamide/2X SSC in PBS 1X at RT 30 min
- Switch to 70% Formamide/2X SSC just before the denaturation

3. Denaturation

[0165]

- denaturate for 4 to 5 min, in particular 5 minutes, at 75°C
- Keep on ice until probe is ready

4. Probe design and preparation

[0166]

- size of probes ~1 kb or shorter
- direct use of fluorescence probes is preferred

5. Pre-hybridisation

[0167]

- For 1 slide, mix in 25 µL final volume:
  - 40 ng fluorescent probe (final concentration: 1.6 ng/µl)
  - 400 ng salmon sperm DNA (from 100 ng/µl solution)
  - Buffer: 10% dextran sulfate/50% Formamide/2X SSC in PBS 1X
- Incubate the mix 10 min at 80°C in the dark
- Pre cool 30 min at 37°C in the dark before incubating slides

6. Hybridization

[0168]

- Drop 25 uL of pre-hybridization mix on square parafilm
- Invert slides on drops
- Incubate 15 hours at 37°C on a heating metal block in the dark (cover with plastic top to set the dark position)

7. Washes and mounting

[0169]

- wash 2 times for 2 min in 3 mL 2X SSC at RT
- cover with black top to protect from light.
- wash 2 times in 1X SSC at RT, then 2 times in 0.1 X SSC at RT,
- wash 2 times in PBS 1X at RT (all in the dark).

8. 3D-FISH

[0170] The following protocol is optimized for 3D Z-scanning of mammalian cells using a confocal spinning disk microscope. Z-stacks of 200nm. 3D reconstruction using the IMARIS (Bitplane software).

- Incubate 1 h in Hoechst 33342 10 ug/mL final
- Wash 5 times in PBS 1X
- Mount on 20 uL PBS 1X on 70% EtOH cleaned and dried slides.
- Keep the slides in Dark clean box at RT until confocal analysis.

[0171] Until this point the procedure allows the labelling of mt DNA and RNAs. With the next steps it allows the additional labelling of mitochondrial or cellular proteins.

9. 3D-FISH coupled Immunofluorescence

[0172] The following protocol is optimized for 3D Z-scanning of mammalian cells using a confocal spinning disk microscope. Z-stacks of 200nm. 3D reconstruction using the IMARIS (Bitplane software).

- Incubate the slide with BSA 5% in PBS 1 X 1 h RT in the dark.
- Wash 2 times in PBS 1X
- Incubate with Primary antibody in BSA 1%, PBS 1X, 1 h RT dark
- Wash 3 times in PBS 1X
- Incubate with Secondary antibody in Hoechst 33342 10 ug/ml final, BSA 1%, PBS 1X, 1h RT dark
- Wash 5 times in PBS 1X
- Mount on 20 uL PBS 1X on 70% EtOH cleaned and dried slides.
- Keep the slides in dark clean box at RT until confocal analysis.

[0173] Are also disclosed nucleic acid molecule(s) suitable for use as probe(s) as defined herein and especially designated as the "first probe" for use in the process of the present disclosure or invention.

[0174] Preferably, such a nucleic acid molecule is suitable for use as a probe suitable for *in situ* hybridization targeting the

genomic DNA.

[0175] Therefore, is disclosed a nucleic acid molecule suitable for use as a probe, hybridizing with a target region in a eukaryotic genomic DNA, wherein said target region comprises a nucleic acid sequence which has no identified corresponding annealing RNA in the metabolically active cell containing said eukaryotic genomic DNA and therefore remains RNA-free during transcription and replication of said DNA genome. According to a particular embodiment, such a nucleic acid molecule hybridizes with said RNA-free nucleic acid sequence, and has the same length as said RNA-free nucleic acid sequence or is longer.

[0176] Nucleic acid molecule(s) or probe(s) disclosed above and herein with respect to a method according to the present disclosure are themselves part of the object of the present disclosure. They are prepared and/or used as either a single stand molecule or a double strand molecule of complementary sequences.

[0177] Are disclosed nucleic acid molecules that are specific for a segment of non transcribed mitochondrial gDNA and comprises or consists of :

1. i. the nucleic acid having the sequence of any one of SEQ ID N°1 to SEQ ID N°16 or,
2. ii. the nucleic acid that has a sequence that is complementary of any one of SEQ ID N°1 to SEQ ID N°16 or,
3. iii. a fragment of (i) or (ii) or,
4. iv. a nucleic acid that has at least 80% identity with the nucleic acid sequence of any one of SEQ ID N°1 to SEQ ID N°16 or the nucleic acid sequence that is complementary of any one of SEQ ID N°1 to SEQ ID N°16 or fragments thereof,

said nucleic acid molecule being either a single stand molecule or a double strand molecule of complementary sequences.

[0178] A nucleic acid molecule of the invention is as defined in claim 13 or 14.

[0179] Is also disclosed a kit for carrying out *in situ* hybridization on fixed cells, comprising a so-called first probe, consisting of a nucleic acid molecule, of the present disclosure, and comprising optionally a so-called second probe, consisting of a nucleic acid molecule, of the present disclosure, hybridizing with a RNA molecule.

[0180] Is also disclosed a kit comprising a probe, consisting of a nucleic acid molecule, suitable for *in situ* hybridization targeting the genomic DNA as disclosed herein, and comprising optionally a so-called second probe, consisting of a nucleic acid molecule, of the present disclosure, hybridizing with a RNA molecule.

[0181] A kit of the invention is as defined in claim 15.

[0182] According to a particular embodiment, said kits further comprise probes, consisting of nucleic acid molecule(s), and/or antibody(ies) for additionally detecting protein(s).

[0183] Kits to label and detect in a same cell or tissue DNA and optionally RNA and/or proteins are useful for the detection of the occurrence of initiation events in the genomic DNA replication in eukaryote cell(s) or tissue(s).

[0184] Said kits can further comprise instructions for use in a process for detecting the occurrence of initiation of replication events in genomic DNA in a eukaryotic cell, according to a methods as disclosed herein.

[0185] Said kits can further comprise reagents necessary for carrying out such a process, as disclosed herein.

[0186] Said kits can further comprise material, e.g measurement material, data carrier(s), recording support(s), to collect or analyze the data measured by a process according to the present disclosure or invention.

[0187] The present invention is of particular interest for analyzing the processing of DNA, RNA or metabolites in cell(s) or tissue(s), and/or analyzing the dynamics of said cell(s) or tissue(s), and/or detecting specific diseases.

[0188] The invention is of particular interest for providing means useful for the analysis, detection and optionally subsequent diagnosis of mitochondrial disease(s) or neoplasic diseases(s) or cancer(s). The invention is also of particular interest for analyzing, detecting and optionally subsequently diagnosing mitochondrial disease(s) or neoplasic diseases(s) or cancer(s).

[0189] Uses according to the present invention are defined in claim 16.

[0190] With respect to the interest of analyzing or detecting the occurrence of initiation of replication events in a context of mitochondrial dysfunction, it is knowledgeable to notice that the physiology and metabolism of mitochondria impact not only in the production of cellular energy (ATP) but also in cell growth, cell differentiation, cell signaling and death (apoptosis). Thus, mitochondrial misfunction is associated with a variety of diseases (cancers, myopathies, neuropathologies, infections), and with the ageing process.

[0191] Mitochondrial diseases encompass cardiomyopathy, neuropathy, Retinitis pigmentosa, encephalomyopathy, hepatopathy, hypotonia, Renal tubulopathy, Leigh syndrome, Barth syndrome, optic atrophy and Ataxia, Leukodystrophy, Diabetes, Kearns-Sayre syndrome.

[0192] Mitochondrial dysfunctions can lead to or are involved in type 2 diabetes, Parkinson, Alzheimer, Atherosclerotic heart disease, stroke or cancers.

[0193] The method of the invention, the probes described herein and kits encompassing said probes or permitting to carry out the method of the invention are useful in clinical diagnosis protocols by contributing to means necessary to identify and/or to class diseases associated with mitochondrial dysfunctions according to the default in mitochondrial mtDNA processing, e.g. transcription and replication, or in mtDNA content. This includes genetic diseases (mtDNA depletion diseases) and cancers. The same can be also used to identify mtDNA depletion induced by clinical treatment (i.e., long term treatment with anti-HIV nucleoside analogues deplete mtDNA) or impaired or abolished initiation of mtDNA replication.

[0194] The method of the invention, the probes or nucleic acid molecules described herein and kits encompassing said probes or nucleic acid molecules or permitting to carry out the method of the invention can be used in the analysis and detection of mitochondrial disease(s) or disease(s) resulting from mitochondrial dysfunction(s) or impairment, or disease(s) resulting in mitochondrial dysfunction(s) or impairment.

[0195] The method of the invention is applicable to all eukaryotic cells, including human, mouse, insect, yeast, fish or plant cell as a diagnosis tool or a biotechnological tool for exploring the functions of said cells.

[0196] The method of the invention, the probes or nucleic acid molecules described herein and kits encompassing said probes or nucleic acid molecules or permitting to carry out the method of the invention can also be used in the analysis and detection of neoplasic diseases(s) or cancer(s).

[0197] The invention provides means useful for the detection and diagnosis of neoplasic or tumoral cell(s) or tissue(s), and especially to distinguish said cell(s) or tissue(s) among healthy cell(s) or tissue(s).

[0198] The present invention is also of particular interest for testing the cytotoxicity of organic and chemical compounds, especially drugs.

[0199] Other examples and features of the invention will be apparent when reading the examples and the figures, which illustrate the experiments conducted by the inventors, in complement to the features and definitions given in the present description.

LEGENDS OF THE FIGURES

[0200]

Figure 1. A modified 3D-FISH reveals a perinuclear mitochondrial subpopulation (a) 3D reconstruction of a dividing HeLa cell shows perinuclear distribution of the mitochondrial subpopulation labeled with the mt DNA probe mix mTOT (red). The entire mitochondrial network is labeled with MitoTracker (green) and the nucleus with Hoechst 33342 (blue). On the right, magnification shows MitoTracker labeling (bottom), mTOT (middle) and merge (top). Scale bar=10 um. Zoom scale bar=3 um. **(b)** 3D FISH-reconstructed HeLa cells labeled with the mTOT probe (red), with or without nuclease treatment (specified on the top of each panel; the arrow indicates that the second nuclease was added after 1h incubation with the first nuclease). Scale bar=10 um. **(c)** Fluorescence intensity quantification of mTOT, with or without nuclease treatment, indicated on the X-axis ("then" indicates that tof he second nuclease was added after 1h incubation with the first nuclease) n=30. T-test, compared to untreated cells, (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

Figure 2. Spatio-temporal distribution of DNA processing mitochondrial subpopulations (a) 3D-FISH of HeLa cells with 14 individual mtDNA probes (red), each covering a portion of the entire mitochondrial genome. Nuclei (blue) are labeled with

Hoechst 33342. Scale bar=10 um. The probe number, and the mitochondrial gene(s) covered by the probe are indicated on the top and on the bottom of each panel, respectively. The central panel is a schematic representation of the mt genome (external circle) and of single genes within, at scale. tRNA genes are indicated with the corresponding letter. All genes are located on the H-strand, with the exception of *ND6,* located on the L-strand. The ribosomal RNAs (16S and 12S) are in dark grey and are slightly shifted out of the circle. The D-loop region that contains the origin of replication of the H-strand (O_H) and the promoters of both the H and the L strands (PH1-PH2, and PL, respectively) is shown in black and shifted out of the circle. The inner circle represents the position of each individual probe (see coordinates in Tables 1 and 2); some probes overlap by a few dozen of bases with the neighbor probes. The 14 individual probes cumulatively cover the complete mt genome (b) Fluorescence intensity quantification of 3D-reconstructed HeLa cells labeled with each of the individual 14 mtDNA probes, indicated on the X-axis, untreated or treated with either DNase I and RNase A. Key genes and regulatory regions are indicated on top. n=30, from three independent experiments. For each probe, the t-test was performed for nuclease-treated versus untreated cells; (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$. A further probe, called ND6, which covers the gene with the same name located on the L-strand, was also tested. For most probes, the labeling decreases dramatically or almost disappears after RNase treatment, indicating that the labeling essentially target RNA molecules. A partial or total reduction of the labeling results from DNase treatment of probes 4, 8 and 13, indicating that these probes target also mtDNA. Probes 4 and 13 cover regions of the mt genome that contains replication origins (O_L and OH, respectively), indicated on the bottom. **(c)** 3D FISH-reconstructed IMR90 primary fibroblasts labeled with the 14 individual mtDNA probes (red); legend elements as in (a). **(d)** Fluorescence intensity quantification of the 14 individual mtDNA probes; legend elements as in (b). n=30 from three independent experiments.

Figure 3. Detection of regions containing mitochondrial replication origins. (a) Schematic localization of DNA and RNA labeling by mt probes used in 3D-FISH assay. DNA labeling is observed on three regions (not at scale), two of which correspond to the major origins of replication of the mt genome (O_H and O_L, probes 13 and 8, respectively), and the third (probe 8) to a additional O_L origin of replication, that was previously identified[13]. The distances between mt origins are indicated. **(b)** Characterization of the O_H region analysed by 3D-FISH with progressively shorter probes. The region covered by each probe is shown on the left panel that also indicates the main genetic elements present in the region (LSP= light-strand promoter; P_{H1} and P_{H2} stand for heavy-strand promoter 1 and 2, respectively; O_H= origin of H strand DNA replication; F=$tRNA^{Phe}$; 12S=12S rRNA, P= $tRNA^{Pro}$). Only the probe mREP covers a DNA region that is not transcribed (indicated as RNA-free), while probes 13 and 13-1 cover also transcribed regions. Panels on the right show by 3D-FISH the localization of mitochondrial entities labeled with each of the probes (red); nuclei in blue (Hoechst 33342). Scale bar=10 um. **(c)** 3D FISH-reconstructed HeLa cells labeled with mREP (red) and mTRANS (green) probes, with or without nuclease treatments. Scale bar=10 um (upper panels), and fluorescence intensity quantification (lower panels). Values of mREP in the presence of DNaseI and of mTRANS in the presence of RNaseA correspond to background. n=30 from three independent experiments: t-test for nuclease-treated versus untreated cells; (***) $p< 0.001$. **(d)** 3D-FISH coupled IF with mREP (red) probe and anti-Poly (green). On the right, fluorescence intensity quantification of mREP-positive and mREP-negative Poly labeled areas. Examples of the respective areas are shown on the bottom, circled, lower panels: Poly; upper panels: merge. n=300 from three independent experiments; T-test (***) $p< 0.001$. (e) 3D-FISH coupled IF with mREP (red) probe and anti-TFAM (green). On the right, fluorescence intensity quantification of mREP-positive and mREP-negative TFAM-labeled areas. Examples of the respective areas are shown on the bottom, circled, lower panels: TFAM; upper panels: merge. n=300 from three independent experiments; T-test (***) $p< 0.001$.

Figure 4. 3D-FISH reveals mitochondria lacking transcription and initiation of replication activity (a) 3D-FISH reconstructed HeLa cell also immunolabeled for the mitochondrial protein TOM22 reveals simultaneously mtDNA, mtRNA and the mitochondrial network. Right panel: mREP; middle panel: mTRANS; left panel: merge. In the left panel, mitochondrial transcripts (mTRANS, red, see arrow) are detected as independent entities in the mitochondrial network (anti-TOM22, green), whereas the mt initiation of replication units (probe mREP, blue) essentially colocalize with the transcript carrying units (merge, purple, see arrowheads). **(b)** Lack of mREP and mTRANS labeling in HeLa *rho*° cells. 3D-IF and 3D-FISH reconstructed HeLa *rho*° cells (whose mitochondria do not contain DNA) with independent labelling anti-TOM22 (green), left panel; mREP (red), middle panel; and mTRANS (green), right panel. Scale bar=10 um. (c) Fluorescence intensity quantification of the experiment above, n=30 from three independent experiments (lower panels). Controls were untreated and nuclease-treated HeLa cells. The estimation of mtDNA content was performed by RT-qPCR. n=3; T-test compared mutant and treated cells to untreated HeLa cells; (***) $p < 0.001$.

Figure 5. Characterization of the 3D-FISH labeling (a) 3D-FISH reconstructed HeLa cells labeled with: the mTOT probe (red) at saturating concentrations (200 ng; left panel); co-FISH of mTOT probe (red) and of the Hs Alu probe, for the nuclear Alu sequence, (green) (middle left panel); mTOTAr, that consists in mTOT without probes 1, 2, and 14 that cover the mt ribosomal genes, untreated (middle right panel), and treated with RNAse A (right panel). Scale bar=10 um. **(b)** Fluorescence intensity quantification of mTOT and mTOTAr with and without nuclease treatment. n=30 from three independent experiments; T-test, compared to untreated cells, (*) $p < 0.05$; (**) $p < 0.01$; (***) $p< 0.001$.

Figure 6. Real-time quantitative PCR of individual mitochondrial genes. (a) Expression levels of the individual mitochondrial genes in Hela cells. Mean of 3 experiments ± standard deviation. **(b)** Relative gene expression of *16S* and *ND1* compared to *12S (12S* was arbitrarily indicated as 1) in HeLa cells and in IMR-90 human primary fibroblasts.

Figure 7. Semi-quantitative analysis of the proportion of mitochondria labeled with each of the 14 mt DNA probes. (a) 3D-FISH coupled with immunofluorescence. Reconstructed HeLa cells labeled with the mtDNA probe indicated (red) and anti-TOM22 (green). The number in each panel indicates the probe used. **(b)** Percentage of 3D-FISH labelled mitochondria in the total mitochondrial population (for each probe, co-labelling of 3D-FISH and anti-TOM22). For each probe, n=30 cells; three independent experiments. **(c)** Intensity of fluorescence in TOM22-labelled mitochondria, calculated for each probe by multiplying the intensity of fluorescence (fi) by the percentage (p) of FISH labeled mitochondria (= fi x p). Each value indicates the relative amount of transcripts carried by the TOM22-labelled mitochondrial population.

Figure 8. 3D-FISH on the 12S region using an alternative probe. (a) 3D-FISH of two HeLa cells with the probe 14-1. Scale bar=10 um. **(b)** Quantification of the fluorescence intensity with the probe 14-1 (data for probes 1 and 14 are from Fig. 2b) in HeLa cells, indicating that the labeling is dramatically lower for the 12S (probes 14 and 14-1) than for the 16S (probe 1) containing region. n= 30 from three independent experiments.

Figure 9. Perinuclear distribution of mitochondria labeled with the individual mtDNA probes. The histogram shows the perinuclear localization, defined as the region within 2µm from the nuclear border, of mitochondria labeled with the probes indicated on the X-axis, in HeLa ells and in IMR-90 primary fibroblasts (n = 30; from three independent experiments). Mean ± SEM.

Figure 10. Co-labeling with several mtDNA probes. (a) 3D FISH-reconstructed HeLa cells co-labeled with probes 2 (green) 3 (red) and 4 (blue), upper panels; and merge with 5x magnification of three distinct regions (lower panels). The probe used is indicated with on top. Scale bar = 10µm. **(b)** percentage of co-localization of the various probes; n=30 cells from three independent experiments. Co-labeling shows that most mitochondrial entities labeled with probe 2 *(16S RNA)* are also labeled with probe 3 *(ND1),* the following gene on the H-strand, but not with probe 4 that covers *ND2*, the further gene on the H-strand. Percentages of co-labeling are measured taking into account the total intensity of fluorescence specific to each probe. Therefore, percentages of co-labeling between probe pairs (as probes 2 *vs* 3 compared to probes 3 *vs 2)* may be different. **(c)** Scheme of the position of probes on the mitochondrial genome, as from Figure 2a. **(d)** 3D FISH-reconstructed HeLa cells co-labeled with probes 7 (red) and 9 (green), upper panels; with probes 10 (red) and 9 (green) middle panels; with probes 12 (blue) and 14 (green) (lower panels). The probes used are indicated on each panel. Merge are on the right panels. Scale bar=10 um. **(e)** Percentage of co-localization of the various probes; for each pair of primers, n=30 cells, from three independent experiments. Note the high intensity of labeling with probe 7 at some mitochondrial entities, which may explain the reduced percentage of co-labeling with probe 9. Note also the strong and distinct labelling with probe 10 compared to the more diffuse labeling with probe 9, as well as the different spatial distribution of the two types of labelling. These differences may explain the limited co-localization between probes 9 and 10. **(f)** Summary of co-localization results. On the X-axis is indicated the tested probe. On the Y-axis is reported the intensity of co-labelling with the probe indicated on the segment of the column. These Y-values represent the percent of colabeling multiplied by the fluorescence intensity signal of the tested probe.

Figure 11. Analysis of probes to label initiation of mt DNA replication. (a) 3D FISH-reconstructed HeLa cells labeled with probe 13-1 (red) either untreated or treated with nucleases, as indicated. For each condition, two different cells are shown (upper and the lower panel, respectively). Scale bar=10 um. **(b)** Fluorescence intensity quantification of 3D-reconstructed HeLa labeled with either probe 13, probe 13-1, or probe mREP, indicated on top. The X-axis indicates whether cells were untreated or treated with either DNase I and RNase A. For each probe and for each condition, n=30 from three independent experiments. T-test, compared to untreated cells, (**) $p < 0.01$; (***) $p < 0.001$. Values for mREP, shown here for direct comparison, are as in Figure 3c. Treatment with DNaseI induces an increase in the labeling with probe 13-1, indicating that a portion of this probe is normally inhibited from binding because of the presence of a DNA-associated structure. However, this is not the case with the more extended probe 13. The reason for such increase is not clear and the sequence involved has not been identified.

Figure 12. mREP labels initiation of mtDNA replication. (a) Labeling with the mREP probe anticipates oxydative stress-dependent increase of the mtDNA content. Kinetics of 3D-FISH coupled immunofluorescence (IF) labeling of H2O2-treated HeLa cells; anti-TOM22 (green, upper panels) and either mREP (red, middle panels) or mTRANS (red, lower panels) probes. Scale bar=10 um. **(b)** Fluorescence intensity quantifications. TOM22; mREP and mTRANS. n=30 from three independent experiments. mtDNA content estimation by qPCR (12S region); expression of the CytC transcript, coded by a nuclear gene, by qPCR. n=3. T-test (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$. Ctrl, untreated. **(c)** mREP probe largely co-localizes with BrdU-positive mitochondria. 3D-FISH-coupled IF reconstructed HeLa cells labeled with mREP (green); anti-BrdU (red), and merge. Scale bar=10 um. (left panels). 5x and 20x magnification are shown on right panels. Arrows indicate colabeling or absence of colabeling (1 = mREP signal fully covers BrdU; 2= partial overlap, and 3=no overlap between mREP and BrdU signals).

Figure 13. mREP sequences and alignments. (a) Human mREP sequence (SEQ ID No 1), having coordinates 446-544 on the human mtDNA sequence disclosed under accession number NC-012920.1 (NCBI or GenBank or MITOMAP accession number) (b) mREP (SEQ ID No 1) - Human polymorphism - variations. Positions where polymorphisms, e.g. nucleotide(s) variation(s), might be found are put on a black background. One skilled in the art can identify variable positions in the mREP sequence from the above-mentioned indications (c) Human mREP alignments with the corresponding sequences (SEQ ID No 2 to 16) in different organisms (Accession numbers disclosed in said figure are NCBI accession numbers http://www.ncbi.nlm.nih.gov)/).

Figure 14. (a) Experiment disclosing a tight association of mt initiation of DNA replication and mt transcripts in healthy primary cells but not in cancer-derived cell-lines: 3D FISH-reconstructed HeLa cell (upper panel) and primary human fibroblast IMR-90 (lower panel) also immunolabeled for the mitochondrial protein TOM22. Mitochondrial transcripts (probe mTRANS, red) are detected as independent entities in the mitochondrial network (anti-TOM22, green), whereas the mt initiation of replication units (probe mREP, blue) essentially colocalize with the transcript carrying units (merge, purple). Right panels represent details of the left panels (5X magnification). Note that in primary fibroblasts mitochondrial transcripts essentially co-localize with initiation of replication units and are almost not detected as independent units. Scale bar=10 µm. **(b)** Expression level of mitochondrial transcripts in cancer cells **(c)** Expression level of mitochondrial transcripts in healthy cells. **(d)** Quantification of co-labelling of either mTRANS or mREP with anti-TOM22 in HeLa cells and IMR-90 primary fibroblasts. For each condition **(d)(A)** Quantification of co-labelling of mTRANS with mREP (mitochondrial entities carrying transcripts and being involved in initiation of replication), **(d)(B)** and of co-labelling of mREP with mTRANS (mitochondrial entities involved in initiation of replication that also carry transcripts) in Hela cells (left panel) and IMR-90 primary fibroblasts (right panel). For each condition, n=30 cells; three independent experiments.

EXAMPLES

A. MATERIALS AND METHODS

[0201] Cells and culture conditions. Human HeLa cells and IMR90 primary fibroblasts (purchased from ATCC) were grown in MEM medium with 10% foetal bovine serum (FBS), HeLa *rho°* cells in DMEM medium with 10% FBS 1 mM sodium pyruvate and 0.2 mM uridine, at 37°C and in the presence of 5% CO2. Cells cultures were split at regular intervals for different experiments as required. IMR-90 cells were at passage 15. Culture under low oxidative stress were treated with 50 uM H2O2 for the time indicated.

[0202] Reagents and Antibodies. BrdU, anti-TOM22 Atto488, and Hoechst 33342 were purchased from Sigma; anti-BrdU antibody from BD Biosciences; MitoTracker® Green FM, and secondary antibodies (Goat anti-mouse antibodies and Goat anti-rabbit antibodies Alexa® Fluor 555 or Alexa® Fluor 488 conjugated) were purchased from Invitrogen.

[0203] Immunofluorescence (IF). Cells plated on slides were fixed with 2% PFA and permeabilized with 0.5% Triton X-100. The slides were incubated in blocking buffer (BSA 5%; PBS 1x) for 1 hr then with the primary antibody for 1 hr. A secondary anti-mouse or anti-rabbit antibody Alexa® Fluor 555 or Alexa® Fluor 488 conjugated was applied. The DNA was stained with 10 µg/ml Hoechst 33342 and the image analysis was carried out using Perkin-Elmer Ultraview RS Nipkow- spinning disk confocal microscope. For MitoTracker analysis, 200 nM MitoTracker® Green FM were added to fixed/permeabilized cells and incubated for 1 hr.

[0204] Probe labeling and denaturation. The DNA probes for FISH were labeled by nick translation of PCR products, incorporating Atto425-dUTP, or Atto488-dUTP, or Atto550-dUTP, using commercial kit (Atto425/Atto488/Atto550 NT Labeling kit, Jena Bioscience). 40 ng of labeled probes were mixed with 400 ng of sonicated salmon sperm DNA (Sigma) and hybridization buffer (50% formamide, 10% dextran sulfate, in 2x SSC pH 7.0). The hybridization mix was denatured at 80°C for 10 min then kept at 37°C for 30 min.

[0205] Modified 3D-FISH and 3D-FISH coupled IF. Cells plated on slides were fixed with 2% PFA and permeabilized with 0.5% Triton X100. Cells were then incubated in 50% formamide (pH=7.0)/2x SSC for 30 min at RT, and denaturated in 70% formamide/2xSSC for 5 min at 75°C. Hybridization was done with 40 ng of probe (single probe or mix) for 16 hrs at 37°C. After washing the slides in 2xSSC, 1xSSC then 0.1 xSSC, the DNA was stained with 10 µg/ml Hoechst 33342, and 40 ng of probe (single probe or mix) and the image analysis was carried out using spinning-disk Perkin Elmer confocal microscope. Experiments at saturation were performed with 200 ng of probe. When required, fixed/permeabilized cells on slides were treated with RNAseA

(100 µg/ml, Roche), or RNAseH (100 U/ml, NEB) or DNAseI (100 U/ml, Invitrogen) for 1 hr at 37°C. When more than one nuclease were used, the enzymes were either added simultaneously or the second nuclease was added after incubation with the first nuclease, followed by three washes with PBS, and further incubation for 1 hr at 37°C. For 3D-FISH coupled IF, after hybridizaton and 0.1xSSC wash, the immunofluorescence procedure was applied.

[0206] BrdU incorporation. Cells plated on slides were incubated for 10 min in the presence of 100 µM BrdU, then immediately fixed in 2% PFA (10 min), treated for 10 min with 4N HCl and 0.5% Triton X-100, and neutralized for 30 min by 100 mM sodium borate. Cells were blocked in 5% BSA in PBS and permeabilized with 0.5% Triton X100[26]. BrdU was detected by immunostaining with anti-BrdU antibody. The DNA was stained with 10 µg/ml Hoechst 33342, and the image analysis was carried out using spinning-disk Perkin Elmer confocal microscope.

[0207] FISH coupled BrdU. Cells plated on slides were fixed with 2% PFA and permeabilized with 0.5% Triton X100. Denaturation was performed using buffer containing 10 mM Tris HCl pH 8.0, 50 mM KCl, 5% glycerol at 95°C for 8 min. The slides were washed in 0.1xSSC and series dehydrated in 70%, 90%, and 100% ethanol and finally air-dried[27]. Hybridization was done overnight at 37°C. After washing the slides in 2xSSC then 0.1xSSC, the slides were incubated in blocking buffer (BSA 5%; PBS 1x) for 1 hr, then incubated with mouse anti BrdU antibody for 1 hr. A secondary anti-mouse antibody Alexa® Fluor 555 or Alexa® Fluor 488 conjugated was applied. The DNA was stained with 10 µg/ml Hoechst 33342 and the image analysis was carried out using spinning-disk Perkin Elmer confocal microscope.

[0208] Confocal acquisition, 3D-reconstruction and quantification. Confocal acquisitions were performed using a spinning-disk Perkin-Elmer Ultraview RS Nipkow Disk, an inverted laser-scanning confocal microscope Zeiss Axiovert 200M with an Apochromat 63x/1.4 oil objective and a Hamamatsu ORCA II ER camera (Imagopole, PFID, Institut Pasteur). Optical slices were taken every 200-nm interval along the z-axis covering the whole depth of the cell, at resolution of 1.024/1.024 pixels. Three-dimensional reconstruction was achieved using the IMARIS software (Bitplane). Fluorescence quantification was done using a single-imaging frame collection and ImageJ 1.34-s software (post-acquisition analysis). The perinuclear location of FISH-labelled organelles corresponds to mitochondria located within 2 µm from the nuclear surface. The percentage of perinuclear 3D-FISH mitochondria was calculated on the total 3D-FISH labelling. Quantification of mREP-positive and mREP-negative mitochondria was performed on either Poly or TFAM immunolabeled areas. For each condition, 300 samples of identical surface were analysed. Co-localization studies were done with ImageJ JACoP plug-in[28]

[0209] Statistical analysis. The significance of differences between data was determined using Student's *t* test for unpaired observations.

[0210] RT-qPCR. Total RNA was isolated from HeLa cells and IMR90 primary fibroblasts using a RNAeasy Mini kit (Qiagen) and a RNAeasy Micro kit (Qiagen), respectively. The total RNA was treated with DNaseI (Qiagen), then reverse-transcribed using Superscript® III Reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed using Power Sybr Green PCR Master Mix (Applied Biosystems) and the rate of dye incorporation was monitored using the StepOne™ Plus RealTime PCR system (Applied Biosystems). Three biological replicates were used for each condition. Data were analyzed by StepOne Plus RT PCR software v2.1 and Microsoft excel. TBP transcript levels were used for normalisation of each target (=ΔCT). Real-time PCR CT values were analyzed using the $2^{-\Delta\Delta Ct}$ method to calculate the fold expression (all (A^2CT)method)[29]. Custom primers were designed using the Primer3Plus online software (http://www.bioinformatics.nl/cgi-bin/primer3plus.cgi). Primers used for amplification are available upon request.

B. RESULTS

Identification of mitochondrial subpopulations by improved FISH

[0211] To gain insight into the dynamics of mitochondrial DNA and RNA inside the organelle, the inventors have developed a novel approach that labels simultaneously mtDNA and mtRNA in human cells by improving fluorescence *in situ* hybridization (FISH), and performed 3D confocal acquisitions (3D-FISH). Since proteins are not destroyed during this treatment, and in contrast to existing protocols, 3D-FISH have been coupled to immunofluorescence. Hence, it was possible for the first time to monitor mitochondrial DNA, RNA and proteins simultaneously. Moreover, the intensity of fluorescence could be quantified, thereby permitting a relative assessment of these nucleic acids with single-cell resolution. Strikingly, 3D-FISH revealed that the labelling occurred in a distinct fraction of mitochondria, located predominantly in the perinuclear region in single human HeLa cells (Fig. 1

a; 77.78 % ± 1.72% of labelled mitochondria are located within 2 µm from the nuclear border). This result was intriguing since the inventors used probes that cover the entire 16.5 kbp of the mitochondrial genome (mixture of equimolar amounts of 14 probes, called mTOT, Tables 1-2) and expected all mitochondria to be labelled. Treatment of cells with either DNaseI, RNaseA or RNaseH (specific for DNA-RNA hybrids), and combinations of these specific nucleases before hybridization with the mTOT probes showed that 84% of labelling targets RNA, corresponding to the missing signal in the presence of RNaseA (Fig. 1b, c). Moreover, about 23% of labelling corresponded to DNA and/or structured RNA since it was resistant to combined RNaseA and RNaseH treatment. These values cumulated exceed 100% since treatment with some nucleases increases the intensity of labelling, see below. Interestingly, the higher intensity of fluorescence observed in the presence of RNaseH (1.4-fold compared to untreated cells) revealed that removal of the RNA moiety from RNA-DNA hybrids made DNA available for pairing with the fluorescent probe. These hybrids probably correspond to transcripts bound to their DNA template. Treatment of samples with RNaseH and subsequently with DNaseI restored the fluorescence levels of untreated cells (Fig. 1c), confirming that the DNA portion of RNA-DNA hybrids paired with the fluorescent probe after disruption of the RNA moiety. The latter observation, and the apparent absence of effect of the DNaseI treatment, indicated that mtDNA is available in limited amounts for binding with fluorescent probes, unless it is engaged in a local open structure.

Table 1. Coordinates of the probes. The start and end points of probes used for FISH experiments are given on the mitochondrial DNA (NC_012920.1, NCBI or GenBank or MITOMAP accession number, was used as reference). Individual probes are indicated in the upper panel. Mix of more than one probe and their composition are indicated in individual panels below. All probes are oriented in the direction of transcription of the H strand, with the exception of probe ND6 that is in the inverse orientation (transcription on the L strand).

Probe	start	end	size
1	1905	2866	961
2	2842	3554	712
3	3451	4825	1374
4	4805	6129	1324
5	6032	7420	1388
6	7400	8518	1118
7	8498	9824	1326
8	9804	11190	1386
9	11107	12618	1511
10	12513	13517	1004
11	13416	14836	1420
12	14805	16055	1250
13	15778	600	1376
14	501	2024	1523
13-1	16034	521	1041
14-1	650	1598	949
ND6	14658	14180	479
mREP	446	544	98
mTRANS probes 2,6,11			
mTOT probes 1 to 14			
mTOTΔr probes 3 to 13 (rRNA probes excluded)			
human mt genome size : 16554 bp			

3D-FISH labelled transcript profiles of mitochondria

[0212] To investigate the nature of the mitochondrial subpopulations revealed by this approach, the inventors have performed FISH with each of the single 14 probes that were combined in mTOT, in the presence and in the absence of DNAseI or RNAseA.

The inventors have observed that each probe recognized a specific subset of mitochondria and not the entire mitochondrial network (Fig. 2a), indicating that only a subpopulation of mitochondria carries detectable amounts of the target nucleic acid, and that mitochondria may not be functionally alike. Saturation experiments indicated that labelling of a subset of mitochondria was not due to limited concentrations of probe (Fig. 5). Treatment with nucleases showed that all of the probes recognized essentially RNA targets, with the exception of probes 4, 8 and 13, which also recognized DNA (decrease in fluorescence following treatment with DNAsel of 65%, 97% and 47%, respectively).

[0213] The fluorescence measurement of each probe, and its decrease after treatment with nucleases, revealed that 16S rRNA represents the major target of the labelling (probes 1 and 2, Fig. 2b). An intense signal was also observed for ND1 transcript, whose gene is located more downstream on the H-strand (probe 3, see also Fig. 10c). Fluorescence labelling distinctly and progressively decreased with probes that cover the middle and the end of the H-strand, indicating a reduced amount of signal for late *versus* early H-strand transcripts. Quantitative RT-PCR analysis of single transcripts confirmed that 16S is present in a large excess compared to most of the other transcripts (Fig. 6a), as expected[11]. Furthermore, by coupling 3D-FISH for each of the 14 mtDNA probes to immunofluorescence with anti-TOM22, a mitochondrial outer membrane marker that identifies the entire mitochondrial population, the inventors have found that not only 16S rRNA (probes 1 and 2) is present in a larger proportion of mitochondria than are the other transcripts, but also that mitochondria carry larger amounts of this transcript compared to other transcripts (Fig. 7).

[0214] RT-qPCR confirmed the 3D-FISH result that 12S rRNA is present at significantly lower levels than 16S rRNA (Fig. 6a). This was unexpected given that both rRNAs are transcribed from the same promoters, PH1 and to a lower extent PH2[12] (see scheme in Fig. 2a), and that they were reported previously to be produced at similar levels *in vitro*[11]. Although the possibility that the lower signal for 12S rRNA is due to inaccessibility of primers/probes cannot be excluded, this possibility seems unlikely since 12S rRNA levels were confirmed by two different tests, and using three different target regions (see also Fig. 8°. Low levels of 12S RNA were also found in primary fibroblasts (Fig. 2d, Fig. 6b). Figure 2a also shows that in HeLa cells the distribution of labelled mitochondria varies as a function of the mtDNA region tested. Indeed, 16S rRNA is present mainly in mitochondria located in the perinuclear region and in tubular, filamentous mitochondria, whereas transcripts of the last third of the H-strand appear in fragmented mitochondrial entities, distributed more randomly in the cytoplasm. Thus, mitochondria cluster around the nucleus during processing of the 16SRNA, and they spread to the cellular periphery as RNA processing on the H-strand terminates. This finding was confirmed by quantitative analysis of the perinuclear localization of 3D-FISH labelled mitochondrial populations (72.18 ± 2.28% and 72.35 ± 2.66% of labelled mitochondria were located in the perinuclear region with probes 1 and 2 respectively; Fig. 9). Moreover, quantitative analysis revealed preferential perinuclear location (72.87 ± 2.66 %) also for mitochondria carrying abundant transcripts, such as ATP8 (targeted by probe 6). In addition, perinuclear mitochondria with probes recognizing mtDNA (see below) were observed (80.65 ± 1.54% and 73.37 ± 2.24% for probes 8 and 13, respectively, and 65.47± 2.24% for probe 4). In general, the perinuclear localization of mitochondria labelled by 3D-FISH is high in HeLa cells (>50% of the total mitochondrial population). Although a different localization of mitochondrial populations according to the detected type of transcript was also observed in IMR-90 primary fibroblasts, no prevalent perinuclear distribution of mitochondria appears in these cells (30-47%, all probes included; Fig. 2c and Fig. 9). Moreover, mitochondria labelled for various RNAs and DNAs appeared to be more fragmented in IMR-90 fibroblasts than in HeLa cells.

[0215] Mitochondrial transcripts exist as processed transcripts of single genes, and unprocessed polycistronic transcripts[1]. The RNA labelling which we observed with 3D-FISH may represent one or both of these types of transcripts. Experiments involving co-labelling with one or more mtDNA probes helped to distinguish between unprocessed RNA molecules and individual transcripts (Fig. 10). Early transcripts on the H-strand appeared largely as unprocessed molecules, whereas late H-strand transcripts appeared frequently as processed molecules.

FISH signal was not limited by probes concentration.

[0216] To check whether the intensity labelling by 3D-FISH was limited by the amount of probes, the inventors have increased by 5-fold the probe concentration, using 200ng of mTOT, which corresponds to one of the highest values described in the literature for FISH experiments[33]. It was found that increasing probe concentration did not increase the proportion of labelled mitochondria nor the absolute values of the signal (Fig. 5), indicating that the labelling of a subset of mitochondria by 3D-FISH was not due to limited concentrations of the probe.

[0217] The inventors have then co-labelled cells with mTOT and with a probe that targets nuclear Alu sequences[34] (probe Hs Alu) and found that labelling of mitochondrial nucleic acids did not preclude the labelling of nuclear nucleic acids (Fig. 5),

indicating that the accumulation of mtDNA labelling in the perinuclear region was not due to inaccessibility of the probe to the nucleus. This experiment also confirmed that labelling with mtDNA probes was specific to mitochondrial nucleic acids.

[0218] HeLa cells were also labelled with a further mix of probes, called mTOTΔr, that includes all probes present in mTOT with the exception of probes 1, 2 and 14 that cover the rDNA portion of the mt genome. Fig. 5 shows that the intensity of labelling in the absence of rDNA probes was at least as high as with the probe mTOT, indicating that 3D-FISH labels mitochondrial mRNAs even in the presence of large amounts of rRNAs. The inventors have also observed that mTOTΔr-labelled mitochondria were not mostly located in the perinuclear region as it was the case with the mTOT mix. This experiment suggested that different combinations of mt DNA probes label distinct mitochondrial populations. It also indicated that mTOT-labelled mitochondria located in the perinuclear region largely correspond to organelles that contain rRNAs. This notion was confirmed by experiments with individual probes (see Fig. 2a,b) that showed the highest labelling for 16S rRNA.

16S but not 12S rRNAs was present in larger amounts than the other transcripts and was produced by a larger proportion of mitochondria

[0219] 12S and 16S rRNA are transcribed *in vitro* about 10-30 fold more than the other genes on the H-strand[35]. rRNA transcripts are mostly produced from promoter PH1 and terminate at specific regions located downstream of *16S* whereas mRNAs and tRNAs are essentially produced from the PH2 promoter[36], see scheme in Figure 2a. By 3D-FISH, the inventors have found that transcripts containing 16S rRNA (probes 1 and 2) are present in larger amounts than the other transcripts, as expected, but surprisingly this was not the case for transcripts containing 12S rRNA (probe 14, Fig. 2). This finding was confirmed by quantitative RT-PCR (qRT-PCR) analysis of the 16S and 12S rRNAs (Fig. 6a), and by a second fluorescent probe (14-1) in the region of the 12S RNA (Fig. 8). High levels of 16S but not of 12S rRNA were observed also in human primary fibroblasts (Fig. 2d, Fig. 6b).

[0220] High levels of fluorescence were observed, surprisingly, also for probe 3 that essentially covers the *ND1* gene localised downstream of 16S on the H-strand. Although the signal for probe 3 was lower than for 16S rRNA (probes 1 and 2), as expected[35], it was at least twofold higher than for the other genes located downstream of rRNA transcription terminators (Figure 2B). The elevated fluorescence with probe 3, observed in HeLa cells but not in primary fibroblasts (Fig. 2d), seemed due to the targeting of unprocessed transcripts that also contain 16S (see next section). The intensity of fluorescence labelling dropped by at least one half with probes 4 to 7 that recognize the downstream region of the H-strand (genes *ND2* to the *COIII),* and even more with probes 8 to 12 that cover the most downstream region, from *ND3* to *CytB.* Interestingly, low levels of labelling appeared also for probe 13, that recognizes the region with the D-loop. On the L-strand, probe ND6 labels the region containing the *ND6* gene with intensity comparable to that of probes 4-7 on the H-strand. It should be noticed that the fluorescence intensity of transcripts detected with probes 4, 8, and 13 was even lower than the actual value, given that these probes also recognize DNA (Figure 2b).

[0221] The production of large amounts of a given RNA may originate from elevated transcription by individual mitochondria or from a large number of mitochondria implicated in transcription, or both. To investigate this aspect, the inventors have coupled 3D-FISH of each of the 14 mtDNA probes to immunofluorescence with anti-TOM22, a mitochondrial outer membrane marker that identifies the entire mitochondrial population. First, the percentage (p) of co-localization between anti-TOM22 and each probe was assessed. The inventors have found that a large proportion of mitochondria (49-69%) was labelled with probes 1 to 3, and with probes 6-7, while only 19-38% of mitochondria are labelled with the remaining probes (Fig. 7 a-b). These data indicated that a larger number of mitochondria carry *16S* to *ND1* RNAs, and *COII to COIII* RNAs, than of the other mitochondrial transcripts. Then, for each probe the intensity of fluorescence present in TOM22-labelled mitochondria was evaluated, as an indication of the relative amount of transcripts carried by that mitochondrial population. Therefore, for each probe the percentage of labelled mitochondria (value p, see above) was multiplied by the intensity of fluorescence of the probe. It was observed that probes 1 to 3 had the highest values, whereas values were 2.5-fold lower for probes 6-7, and until 20-fold lower for the remaining probes (Fig. 7c). All together, these results indicated that not only 16S and to a minor extent *ND1* RNAs were present in a larger proportion of mitochondria than were the other transcripts, but also that mitochondria carried larger amounts of these than of the other transcripts. Conversely, the remaining transcripts were present in a small proportion of mitochondria where they were also present in little amounts. An intermediary situation was observed for *CoII to* COIIIRNAs (probes 6-7), that were present in a relatively large portion of mitochondria but in small amounts therein.

Labelling of unprocessed and processed transcripts

[0222] An intriguing result of 3D-FISH experiments concerned the high levels of RNA labelling for probe 3 that essentially covers *ND1* (see above). High levels of ND1 labelling may results from PH1 transcription of rRNAs that did not stop at terminators or, alternatively, from a particularly long-lived RNA, although it was not reported that the ND1 transcript was more long-lived than the other mRNA in HeLa cells[37]. In agreement with the first hypothesis, the levels of ND1 labelling (probe 3) were close to those of 16S rRNA (probes 1-2). The inventors have reasoned that large amounts of ND1 RNA may result from leaky termination of transcription from PH1. To check whether ND1 and 16S RNAs labelled by 3D-FISH were present on the same molecules and, more in general, whether RNA labelling by 3D-FISH targeted polycistronic precursor RNAs and/or processed transcripts the inventors have performed 3D-FISH with two or three probes simultaneously. It was found that labelling with probes 2 and 3 mostly overlapped (92 ± 1.4% of probe 2 colocalized with probe 3, and 84 ± 1.9 % of probe 3 colocalized with probe 2, Fig. 10), indicating that 16S and ND1 RNAs were essentially located on the same molecule or on distinct molecules that were present at equimolar amounts on the same mitochondrial entities. This was not the case for probe 4 that targeted the region just downstream of *ND1,* and that colocalized with probes 2 and 3 in only 23 ± 3.2% and 27 ± 3.2 % of cases, respectively, Fig. 10. Thus, if 16S and ND1 RNAs labelled by 3D-FISH were present on the same molecule, then a relevant part of PH1 derived rRNA transcripts may not have stopped at termination signals, but proceeded through ND1, at least in HeLa cells.

[0223] The inventors have performed co-labelling with additional pairs of probes to verify the simultaneous presence of transcripts in mitochondria. It was found that probe 14, that labelled 12S rRNA present at the beginning of the H-strand transcript colocalized with probe 12, that labelled CytB present at the end of the same transcript, in 55.6 ± 7% of cases, indicating that mitochondrial entities showing co-localization either contained the 5' and the 3' end of the PH2-directed transcript, i.e. the complete H-strand transcript, or that 12S and CytB processed transcripts were present in equimolar amounts on the same mitochondrial entities (Fig. 10). It was also observed a large overlap (> 60%) for labelling with probes 7 and 9 that covered close regions localized in the second half of the H-strand, indicating that most mitochondrial entities contained both transcripts or that these transcripts were present on the same molecule. The levels of colocalization decreased with the adjacent probes 9 and 10, that additionally showed a rather diverse spatial distribution, indicating that these probes mostly labelled transcripts located on different mitochondrial entities or on different molecules (Fig. 10). These results are summarized in Fig. 10f that takes into account the percent of co-localization and the total fluorescence intensity of the tested probe. Although just indicative in quantitative terms, these data nevertheless confirms a relevant co-localization of transcripts targeted by probes 2 and 3 (located in the first quarter of the H-strand), and also by probes 7 and 9 (located in the third quarter of the H-strand). On the contrary, data show a scarce co-localization of transcripts targeted by probes 2-3 and 4, and probes 9 and 10, revealing that most of these transcript pairs were not present in the same mitochondria. In conclusion, colocalization experiments strongly suggested that transcripts labelled by 3H-FISH probes represented both unprocessed RNA molecules, in particular the early transcripts on the H-strand, and processed individual transcripts. This proof of principle showed that processed and unprocessed RNA molecules could be identified for any mitochondrial gene of interest using, appropriate pairs of probes in co-localization experiments.

qPCR analysis of mitochondrial transcripts

[0224] To check whether the proportion of the various transcripts detected with 3D-FISH in distinct mitochondrial populations were consistent with the transcript levels of the mitochondria, the inventors have performed qRT-PCR experiments for each mitochondrial rRNA and mRNA gene (Fig. 6a). A direct comparison of 3D-FISH and qRT-PCR data was not suitable since FISH probes used here cover regions larger than a single gene, with the exception of probes 5 and 10 that cover only *COI* and *ND5*, respectively. Nevertheless, for probes 5 and 10 qPCR confirmed 3D-FISH data, and this was also the case for the relative amounts of 16S (probes 1-2) *versus* 12S RNA (probe 14), and also for 16S *versus* most of mitochondrial mRNAs (see above). However, the highly expressed *ATP8* and, to a minor extent, *ND4* were weakly labelled by 3D-FISH (probes 6-7 and 8-9, respectively). The low labelling of probes 6-7 and 8-9 might result from the levels of additional transcripts other than ATP8 and ND4, also identified by these probes (see Table 2).

Table 2. Position of the probe on the human mitochondrial genome. The coordinates of the genetic element present at a given position of the mitochondrial genome (NC_012920.1, NCBI or GenBank or MITOMAP accession number) are indicated in column 1 (data from MITOMAP: http://www.mitomap.org/MITOMAP/HumanMitoSeq). The name of the element itself is indicated either on column 2 or 3 (direct and inverse orientation with respect to the direction of transcription of the H-strand, respectively). In the last three columns is/are indicated the probe(s) that hybridize with the indicated region. Even hybridization of a few nucleotides is indicated.

position	element	element	probe	probe	probe
110-441		Origin H		13	13-1
213-235	CSB1			13	13-1

position	element	element	probe	probe	probe
299-31 5	CSB2			13	13-1
346-363	CSB3			13	13-1
392-445		PL (or LSP)		13	13-1
545-567	PH1		14	13	13-1
577-647	tRNAphe		14	13	
645	PH2		14		
648-1601	12S RNA		14		
1602-1670	tRNAval		14		
1671-3229	16s RNA		14	1	2
3230-3304	tRNAleu (UUR)				2
3307-4262	ND1			3	2
4263-4331	tRNAile			3	
4365-4400		tRNAgln		3	
4402-4469	tRNAf-met			3	
4470-5511	ND2		4	3	
5512-5579	tRNAtrp		4		
5587-5655		tRNAala	4		
5657-5729		tRNAasn	4		
5721-5755	Origin L		4		
5761-5826		tRNAcys	4		
5826-5891		tRNAlys	4		
5904-7745	COI		4	5	6
7446-7514		tRNAser (UCN)			6
7518-7585	tRNAasp				6
7586-8329	con				6
8295-8364	TRNALYS				6
8366-8572	ATP8		7		6
8527-9207	ATP6		7		
9027-9990	COIN		7	8	
9991-10038	tRNAglu			8	
10059-10404	ND3			8	
10405-10469	tRNAarg			8	
10470-10766	ND4L			8	
10760-12137	ND4		9	8	
12138-12206	tRNAhis		9		
12207-12265	tRNAser(AGY)		9		
12266-12336	tRNAleu(CUN)		9		
12337-14148	NAD5		9	10	11
14149-14673		NAD6			11
14674-14742		tRNAglu	12		11
14747-15887	CytB		12	13	
15888-15953	tRNAthr		12	13	

position	element	element	probe	probe	probe
15956-16023		tRNApro	12	13	13-1
16024-191		7SDNA	12	13	13-1

[0225] Moreover, if *ATP8* and *ND4* were being transcribed they might not be labelled by 3D-FISH. Indeed ongoing transcripts formed DNA-RNA hybrids with the template that might not be detected as RNA molecules by 3D-FISH (see Fig. 1c). It is also possible that probes 6-7 and 8-9 had impaired accessibility to RNA molecules. Finally, probe 3 showed high 3D-FISH labelling but *ND1,* its main target, was not expressed at high levels. This apparent discrepancy may be due to the fact that 3D-FISH identified not only full transcripts but also, differently from RT-qPCR, unprocessed and truncated transcripts, and this seemed to be the case for ND1 RNA (see above). In conclusion, although there was a good correlation between 3D-FISH labelling and transcript levels, it should be taken into account that 3D-FISH labelled a variety of RNA molecules (including processed, unprocessed and truncated transcripts), whereas RT-qPCR data were based essentially on full-length transcripts. Moreover, 3D-FISH labelled a subset of mitochondria in single cells, while RT-qPCR detected the transcripts levels of entire mitochondrial and cellular populations. Thus, 3D-FISH revealed RNA molecules that were not necessarily detected by RT-qPCR.

3D-FISH revealed mtDNA initiation of replication

[0226] The inventors have observed above (Fig. 2b) that three probes (13, 4 and 8) detected not only RNA but also DNA. Interestingly, probes 13 and 4 included the regions of initiation of replication of the H- and the L-strand, respectively, suggesting that these probes detected DNA regions engaged in the initiation of replication. Probe 8 included the ND4 region, where an additional origin of replication for the L-strand has been observed using atomic force microscopy[13] and which is expected to be activated less frequently than the two major ones. These three origins are located almost symmetrically on the mt genome, as schematized in Figure 3a. To assess whether DNA labeling by 3D-FISH was associated with initiation of DNA replication, the inventors have investigated the region covered by probe 13 which is unique within the entire mitochondrial genome, as it contains a sequence that is not transcribed, and therefore it should be present only in its DNA form (Fig. 3b). The inventors generated a second probe (13-1) which covered a shorter region than probe 13 and which did not contain genes, and a third probe (mREP) that covered only non-transcribed DNA (Fig. 3b). 3D-FISH with each of the three probes showed that only mREP resulted in fully DNaseI-sensitive and fully RNaseA-resistant labelling (Fig. 3c, Fig. 11) indicating that this probe specifically labelled DNA.

[0227] Cells were also labelled with mTRANS, a combination of three probes (1, 6, and 11) that together covered early, middle and late transcribed regions of the H-strand of the mitochondrial genome. Probes in mTRANS are located almost symmetrically in the circular genome, and do not recognize regions involved in the initiation of DNA replication. 3D-FISH experiments of co-labelling with mREP and mTRANS probes, in the absence and in the presence of nucleases showed that mTRANS recognizes only RNA (Fig. 3c).

[0228] Since the DNA region labelled by mREP is normally present in the genome of all mitochondria, the inventors have reasoned that 3D-FISH labelled only mitochondria where this DNA region was structurally accessible, because of initiation of DNA replication nearby. To assess whether this was the case, immunostaining of DNA polymerase γ (Poly), the enzyme responsible for replication mtDNA, was coupled to 3D-FISH. It was found that the intensity of fluorescence of Poly almost doubled in areas labelled with mREP compared to mREP-negative areas (cells n= 30; mt areas n=300, Fig. 3d). Moreover, mREP-positive mitochondria were associated with higher levels of TFAM immunolabelling compared to mREP-negative mitochondria (Fig. 3e). TFAM is a protein implicated both in transcription of mtDNA and in binding to the mtDNA, and whose levels are correlated with increased mtDNA[14].

[0229] Moreover, treating cells with low concentrations of H_2O_2, which is known to increase the mtDNA content[15], resulted in a transient increase in mREP labelling when the mtDNA content was low (1 h after H_2O_2 treatment). mREP signal returned to original values when the mtDNA content was again elevated in the following 2 hours (Fig. 12 a,b), compatibly with the time necessary to replicate the mitochondrial genome[16]. Furthermore, the inventors have checked BrdU incorporation, an indicator of DNA replication, in mitochondria. It was found that mREP-positive entities co-labelled with BrdU, confirming that mREP labelled mitochondria engaged in DNA replication. Importantly, mREP labelled only a subset of BrdU-positive mitochondria, indicating that mREP did not detect ongoing replication of the complete mt genome but rather a special event of DNA replication. All together these data support the notion that mREP is as a marker of the initiation of mtDNA replication. Whether DNA synthesis proceeds from O_H until the end of the H-strand, or terminates before (i.e. formation of the D-loop) was not defined by this labelling.

mREP labelling precedes the increase of mtDNA content

[0230] The inventors have reasoned that if mREP labelling is an indicator of mtDNA initiation of replication, it should anticipate the increase in mtDNA content. To assess whether is was the case, HeLa cells were treated in culture with low doses (50 µM) of H_2O_2, known to increase the mtDNA copy number and the mitochondrial mass[38]. As expected, treatment with H2O2 resulted in an increase of about 30% of the mitochondrial mass, measured by the intensity of fluorescence of the mitochondrial protein TOM22, and in the increased expression of the mitochondrial biogenesis marker *Nrf1* (Fig. 12 a,b). It was observed that mREP labelling increased by about 70% 1 h after treatment, when the DNA content was low probably due to the stress of the treatment, and returned to control values 3h after treatment when the original mitochondrial DNA content was restored. At 24 h, at high mtDNA content, mREP labelling was as low as in untreated cells. Thus, mREP labelling increased when the mtDNA content was low and returned to original values when the mtDNA content was elevated. Moreover, mREP labelling was followed by a rise in the mtDNA content within 2 hours, compatibly with the time necessary to replicate the mt genome (about 92 minutes for total mtDNA replication[39]), supporting the notion that mREP detected the initiation of mtDNA replication. Under low doses of H_2O_2 we also observed an increase of mitochondrial but not nuclear transcription, measured by labelling with the mTRANS probe and *CytC* expression, respectively.

[0231] Furthermore, the inventors have checked BrdU incorporation, an indicator of DNA replication, in mitochondria. First, it was observed that a large number of mitochondrial entities were labelled with BrdU, corresponding to mitochondria that had replicated or were replicating their DNA. However, only a portion of BrdU-positive mitochondria were also mREP-positive, indicating that mREP labelling targeted only a subset of mitochondria engaged in DNA replication and not the mitochondria whose mtDNA replication was achieved. Importantly, 92.8% ± 3% of mREP labelled mitochondria were BrdU-positive, confirming that the large majority of mREP labelling indeed targeted mitochondria engaged in DNA replication (the other 7.2% of positive-mREP mitochondria could contain BrdU below the limits of the detection). It was also observed that the intensity of BrdU labelling was lower for mREP-positive than for mREP-negative mitochondrial entities (this was visible in isolated mitochondrial entities, right panels in Fig. 12c), in agreement with the limited incorporation of a nucleotide analogue at the beginning, but not at the end, of the replication of the mt genome. These results, and the position of this probe on the mt genome, support the notion that mREP labels events of initiation of replication.

3D-FISH revealed mitochondria lacking transcription and initiation of DNA replication activity

[0232] A variety of nuclear and mitochondrial DNA mutations promote mtDNA depletion and loss [17], and these phenotypes can also be drug-induced, as after treatment with the antiretroviral AZT [18]. Defects in mtDNA have been linked to human diseases [17]. The detection of the mtDNA status has relied on classical molecular biology approaches as PCR, Southern blot and sequencing, and follows the morphological and biochemical diagnosis which suggest defects in mitochondrial function. The inventors have investigated whether 3D-FISH would provide more in-depth information of the mtDNA status in cells with impaired mtDNA content. To do this, the inventors have labelled with 3D-FISH HeLa *rho0* cells, whose mitochondria do not contain DNA[19].

[0233] These cells contained about one third of the mitochondrial mass, identified by TOM22 immunolabelling, compared to regular HeLa cells, but no signal was detected with either mTRANS or mREP, confirming the absence of mtDNA transcription and initiation of replication in these cells (Fig. 4b,c). In contrast, the labelling of HeLa cells with active mitochondria was dramatically different (Fig. 4a), revealing the presence of mitochondria (i) rich in transcripts (mTRANS), (ii) engaged in DNA replication (mREP), (iii) rich in transcripts and also engaged in DNA replication (colabelling mTRANS and mREP), and (iv) not labelled for DNA processing activity (TOM22). Importantly, not only the proportion but also the subcellular distribution of these mitochondrial populations was revealed with this triple labelling. Thus, the 3D-FISH experiments carried out provided direct information of the mtDNA activity, namely transcription and initiation of replication, and this for the entire mitochondrial population within the cell.

C. DISCUSSION

[0234] Understanding the dynamics of DNA transcription and replication within the mitochondrial network is essential to assess mitochondrial function. Mitochondria appear to be homogeneous as a population within single cells, although functional differences have been described for synaptic and non-synaptic mitochondria in neurons[20]. The inventors have devised here a

novel 3D-FISH approach which identifies a variety of mitochondrial populations in single-cells. These populations differ in the intracellular localization, in the relative amount of transcript that they carry and in their engagement in initiation of DNA replication, indicating that mitochondria are more heterogeneous than previously thought in DNA processing activities.

[0235] The 3D-FISH method described herein detected mitochondria rich in a given transcript, that was present as a processed molecule or as polycistronic RNA. Moreover, RNAseH treatment revealed the presence of another class of transcripts (accounting for 38% of additional signal) that were still bound to the DNA template and likely resulted from ongoing transcription. The variety of RNA molecules labelled by 3D-FISH, which also included truncated and misprocessed transcripts, provided more extensive information compared to full-length transcripts detected by RT-qPCR. In this context, although in general a good correlation was found between RNA levels detected with the two approaches, discrepancies between the two tests exposed the presence of alternative mitochondrial transcripts that were not detected by classical methods (e.g., ND1 RNA, see below). Moreover, the 3D-FISH method described herein permitted the detailed investigation of mtDNA dynamics, since it labelled relevant mitochondria in single-cells, whereas RT-qPCR only assessed the transcript levels of entire mitochondrial and cellular populations.

[0236] With these novel tools the inventors have found that, unexpectedly, of the two rRNAs produced from the same PH1 promoter[11], 16S, but to a lesser extent 12S, is abundant in mitochondria. RT-qPCR data confirmed the 3D-FISH finding, and this was the case in both HeLa cells and primary fibroblasts. Importantly, variable levels of 16S *versus* 12S rRNA were detected in liver cells[21] and, in their adenylated form, 16S RNA was more abundant than 12S RNA in the skeletal muscle[22], indicating that lower levels of 12S versus 16S RNA detected with the present analysis, represented a physiological situation.

[0237] Importantly, it was observed that mtDNA processing was not alike in all cell types. It was found that in HeLa cells mitochondria carrying abundant transcripts (16S, ND1 and ATP8) were mainly located in the perinuclear region, whereas the less abundant transcripts of the last third of the H-strand appeared progressively distributed in the cytoplasm and in more fragmented mitochondrial entities. The perinuclear localisation of mitochondria may be required for the nuclear uptake of molecules necessary for intensive mitochondrial transcription and/or DNA replication, or for buffering Ca^{2+} fluctuations from the cytoplasm[23]. However, perinuclear localization of mitochondria has been also described in cells of patients with myopathic and neurodegenerative diseases characterized by mitochondrial dysfunctions[24,25]. In this context, it was interesting to note that perinuclear distribution of most mitochondria, and in particular of the organelles that produce the predominant 16S RNA, was not observed in primary fibroblasts, thus raising the possibility that such localization is associated with mitochondrial impairment. Additional differences characterize mitochondrial DNA in primary cells *versus* cancer-derives cell lines. The 3D-FISH method described herein detected high levels of *ND1* RNA in HeLa cells but not in primary fibroblasts. This transcript was present probably as polycistronic RNA consequent to leaky termination from promoter PH1, indicating that mitochondrial rRNA transcription termination may be altered in HeLa cells.

[0238] Labelling of DNA by the 3D-FISH method described herein appears limited to locally open structures, as in transcription complexes after disruption of the RNA moiety, and in DNA engaged in initiation of replication. Interestingly, a third mitochondrial replication origin previously detected with atom force microscopy and expected to be activated only occasionally[13] was revealed in the experiments that were conducted and its position in the mitochondrial genome defined at a higher resolution. To date, identification of mitochondrial initiation of replication in single cells has been elusive. Importantly, the inventors have defined the characteristics necessary for a probe to specifically mark the initiation of DNA replication, and proposed a specific probe, mREP, which is an efficient marker of initiation of mtDNA replication. Mitochondria engaged in DNA replication could therefore be detected and analysed in cells and under experimental conditions of biological relevance.

[0239] The combination of mtDNA transcription and initiation of replication labelling can provide information on mitochondrial dynamics in a variety of physiological processes. (e.g., the dynamics of mitochondrial DNA transcription and replication during the cell cycle, Chatre & Ricchetti, in preparation). Moreover, the 3D-FISH method which is described herein can provide novel information on alterations of mtDNA dynamics and represents a novel tool which can impact on disease screening related to the mitochondrial function.

D. CONCLUSION

[0240] Mitochondrial DNA (mtDNA) replication and transcription are crucial for cell function, but these processes are poorly understood at the single-cell level. By modified fluorescence *in situ* hybridization, called 3D-FISH, the inventors have identified mitochondria engaged in initiation of DNA replication in human cells. Mitochondria were also distinctly marked according to transcription profiles. Thus, the inventors have documented the existence of mitochondrial subpopulations in single cells

according to the prevalent mtDNA processing activity, indicating that mitochondria may not be functionally alike. Importantly, the inventors have proposed an *in situ* hybridization procedure, and more particularly a 3D-FISH protocol that can be coupled to immunofluorescence, and they were thus able for the first time to monitor mtDNA, mtRNA and proteins simultaneously in single cells and demonstrate significant heterogeneities that have been previously missed. With this approach, novel information can be provided on the dynamics of mtDNA processing during physiological and pathological processes. These findings have implications for the optimization of diagnostic tools for mitochondrial diseases, in particular those involving mtDNA depletion and mtDNA loss.

[0241] Since currently available tools including recent improvements[7], cannot identify mitochondria engaged in DNA replication, they cannot discriminate the transcription profiles of organelles in single cells. Moreover, although sequential RNA and DNA labelling[8], as well labelling of either RNA or DNA, and proteins[9], [10] have been performed, immunofluorescence was not directly coupled to FISH to simultaneously detect proteins and mitochondrial DNA and RNA. Thus, proteins of interest could not be monitored during mtDNA transcription and replication. As a consequence, it remained unclear how mtDNA processing is coordinated among the many organelles present in each cell and whether this process is deregulated in subpopulations during disease. Using a novel approach, the inventors have identified mitochondrial subpopulations engaged in the initiation of mtDNA replication and in RNA processing, and assessed their dynamics in single cells. Theses findings revealed significant heterogeneities within single cells that have been missed previously, and this can impact on how mitochondrial functions are assessed. Mitochondria with altered processing of DNA and RNA, as in diseases involving mtDNA loss, can be identified with this novel approach.

E. APPLICATIONS

[0242] The present invention is of particular interest for analyzing the processing of DNA, RNA or metabolites in cell(s) or tissue(s), and/or analyzing the dynamics of said cell(s) or tissue(s), and/or detecting specific diseases.

[0243] As stated above, mitochondrial misfunction is associated with a variety of diseases (cancers, myopathies, neuropathologies, infections), and with the ageing process, and can be found in a number of mitochondrial diseases.

[0244] Mitochondrial diseases are diagnosed in 11.5/100 000 adults and children per year in the world (~800 000 patients/year), and 1/4 000 (25/100 000) USA children.

[0245] Mitochondrial diseases are difficult to diagnose. Referral to an appropriate research center is critical. If experienced physicians are involved, however, diagnoses can be made through a combination of clinical observations, laboratory evaluation, cerebral imaging, and muscle biopsies. Despite these advances, many cases do not receive a specific diagnosis.

[0246] Most hospitals do not have a metabolic laboratory and therefore can run only the most basic tests. In addition, a single blood or urine lab test with normal results does not rule out a mitochondrial disease. This is true for organic acids, lactic acid, carnitine analysis and amino acid analysis. Even muscle biopsies are not 100% accurate.

[0247] To date, most of the studies on mitochondria are based on molecular biology assays (PCR, qPCR, Southern blot), biochemistry (Western blot, ATP/Reactive Oxygen Species (ROS) / membrane potential detection assays), and electron microscopy (for the mitochondrial ultrastructure).

[0248] For example, current diagnostic tools for the mitochondrial diseases encompass Metabolic Screening in Blood and Urine (complete blood count, lactate, pyruvate, plasma amino acids, liver enzymes, ammonia, urine organic acids...), Metabolic Screening in Spinal fluid (lactate, pyruvate, amino acids, cell count, glucose, protein), Characterization of Systemic Involvement (echocardiogram, ophthalmologic exam, brain MRI, electrocardiogram, audiology testing), Clinical Neurogenetics Evaluation (karyotype, child neurology consultation, fragile X test, genetics consultation).

[0249] There is therefore a need for simple, reliable and fast methods and tools for including in diagnosis protocols of mitochondrial diseases and mitochondrial dysfunctions.

[0250] In addition, fluorescence imaging tracks separately mitochondrial DNA (mtDNA), mitochondrial RNA (mtRNA), by fluorescence *in situ* hybridization (FISH), and proteins, by immunofluorescence (IF), in fixed cells. However, two aspects restrain the potency of fluorescence imaging of mitochondria. First, even using a combination of different imaging procedures (for instance IF and RNA FISH, or IF and DNA FISH, of RNA and DNA FISH), it is not possible to detect in the same cell DNA, RNA and

proteins. This can be due to cross-reaction of chemicals and damages of the samples during the procedure(s). For example, when IF and FISH are combined, FISH provokes damages to the proteins resulting in a reduced fluorescence signal for the proteins that cannot be interpreted correctly. Second, the prior art FISH procedure for the detection of mtDNA contains large DNA probes (i.e. more than 3 kbp), which generate high levels of aspecific staining and thus decrease the overall resolution.

[0251] Therefore, the development of a novel FISH labeling approach of cells that allows the tracking of mitochondrial DNA initiation of replication at the single-cell resolution is of particular interest to reveal dysfunctions at this level. In addition, the present invention further allows the simultaneous detection of mitochondrial RNA, and thus the monitoring of transcription events. Bi-dimensional or three-dimensional imaging can also be performed. Moreover, since the developed FISH procedure does not damage the epitope/antigen, it permits also the simultaneous analysis of mitochondrial and/or cellular proteins. In its 3D version, this technique has been called called 3D-Fluorescence In Situ Hybridization coupled ImmunoFluorescence (3D-FISH coupled IF) and results in a drastic modification of the classic FISH procedure in term of cell fixation, permeabilization, mtDNA probes design, size and fluorescence labeling, cell and DNA probes denaturation.

[0252] Moreover, although the depletion of mitochondrial DNA is currently detected by real time quantitative PCR on biopsies (preferentially muscle biopsies, because of the richness in mitochondria in this tissue and the relatively harmless surgical procedure), these tests only indicate the average mitochondrial DNA content present in the entire mitochondria population, and this in all the cells contained in the biopsy, including non-muscle cells present in the biopsy. By contrast, the present invention enables to detect i) alterations in mitochondrial DNA transcription and replication in any single type of cell, including cells extracted from a buccal sample, which avoids biopsies; ii) the impairment in mtDNA replication and transcription (which are the outcome of the mitochondrial DNA molecule) in a portion or in the totality of mitochondria; iii) the impairment in mtDNA replication and transcription in a specific number or in the totality of tested cells. At present, there are indeed no indications whether mitochondrial depletion disease cells are equally or differently affected in their mitochondrial DNA content and activity. Moreover, the present invention enables to reveal the proportion of mitochondria that display at the same time mitochondrial DNA transcription and replication signal, which indicates efficient cell activity, see figure 14. These data might be of interest or directly useful to follow the progression or even anticipate the progression of mitochondrial diseases.

[0253] The method of the invention, the probes described herein and kits encompassing said probes or permitting to carry out the method of the invention can also be used in the analysis and detection of neoplasic diseases(s) or cancer(s).

[0254] The invention provides means useful for the detection and diagnosis of neoplasic or tumoral cell(s) or tissue(s), and especially to distinguish said cell(s) or tissue(s) among healthy cell(s) or tissue(s).

[0255] Further experiments have shown a tight association of mt initiation of DNA replication and mt transcripts in healthy primary cells but not in cancer-derived cell lines (Fig. 14). In these experiments co-labelling with mTRANS and mREP probes (purple spots, Fig. 14)) was performed to reveal the association of mt initiation of DNA replication and mt transcripts. These experiments demonstrated that in HeLa cells about 92% of mitochondrial transcripts were NOT associated with mt DNA replication), and that in primary fibroblasts only about 27% of mitochondrial transcripts were NOT associated with mt DNA replication (n= 30, from 3 independent experiments). These results were confirmed in other tumor-derived cell lines and primary fibroblasts (data not shown).

[0256] Thus, in tested healthy cells mitochondria that are active in DNA replication were also rich in transcripts (a sign of efficient mitochondrial activity) while in cancer cell lines this occurred only in a small fraction of mitochondria.

[0257] The robust activity of mitochondria in healthy cells was confirmed by high levels of mitochondrial transcripts (10 to 76-fold higher than in the cancer-derived cell line, see Fig. 14b), a result that is in agreement with the reduced mitochondrial activity in cancer cells (known as « Warburg effect »).

[0258] Thus, the co-labelling of mitochondria with mTRANS and mREP (never performed before) measured the efficiency of mitochondrial DNA processing in single cells.

[0259] The method of the invention could therefore be used as an indicator of reduced mitochondrial activity, characteristic of cancer cells.

[0260] The present invention is also of particular interest for testing the cytotoxicity of organic or chemical compounds, especially drugs.

[0261] Indeed, the present invention can be used in particular to assay tissues and organs whose cells are rich in mitochondria,

as it is the case for cardiac and skeletal muscle, as well as liver. Therefore the induction of cytotoxicity by drugs or treatments affecting directly or indirectly these tissues/organs, can be identified and measured by checking mitochondrial DNA transcription and replication. Although lethal cytotoxicity can be evaluated with a number of available tests, the present invention provides for the detection and quantification of non-lethal and transitory cytotoxicity (the one which can have effect on the long term). To this end, the inventors have shown in HeLa cells that a mild cytotoxic agent (50 µM of H_20_2-) known to reduce the mtDNA content[32] results in increase of mREP and mTRANS after a few hours of treatment, and that these events were associated with increase of the mitochondrial mass as well as of the transcription of a mitochondrial biogenesis factor (Fig12B). Since the inventors have used HeLa cells, which are not particularly rich in mitochondria as muscle and liver cells are, this experiment indicates that the invention enables the detection of cytotoxic effects in any type of cells. A more pronounced detection in mitochondria-rich cells is therefore expected.

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SEQUENCE LISTING

[0263]

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Patentkrav

1. Fremgangsmåde til både detektering af forekomst af initiering af replikationshændelser i genomisk DNA, som er mitokondrielt genomisk DNA, og detektering af mitokondrielt DNA-transkription i en eukaryotisk celle, omfattende trinnene:

- at bringe den eukaryotiske celle omfattende mitokondrielt genomisk DNA i kontakt med en første nukleotidsonde, under betingelser, der muliggør *in situ*-hybridisering af den første nukleotidsonde med en målregion i det mitokondrielle DNA-genom, hvor målregionen omfatter en nukleinsyresekvens, som ikke har nogen identificeret tilsvarende annealing RNA i en metabolisk aktiv celle og derfor forbliver RNA-fri under transkription og replikation af det mitokondrielle DNA-genom, hvilken første nukleotidsonde er specifik for et segment af ikke-transkriberet mitokondrielt gDNA og omfatter eller består af:

i. nukleinsyren med sekvensen med et hvilket som helst af SEQ ID N°1 til SEQ ID N°16 eller et komplement deraf eller

ii. en nukleinsyre, der har mindst 80 % identitet med nukleinsyresekvensen med et hvilket som helst af SEQ ID N°1 til SEQ ID N°16 eller et komplement deraf,

hvilket nukleinsyremolekyle er enten et enkeltstrenget molekyle eller et dobbeltstrenget molekyle,

og

- at bringe den eukaryotiske celle i kontakt med mindst en anden nukleotidsonde, der er målrettet mindst et mitokondrielt RNA-molekyle svarende til en transkriberet region af et mitokondrielt DNA-molekyle i cellen, og
- at detektere den første nukleotidsonde, der er hybridiseret til det mitokondrielle DNA, og den anden nukleotidsonde, der er hybridiseret til det mitokondrielle RNA-molekyle.

2. Fremgangsmåde ifølge krav 1, **kendetegnet ved, at** den omfatter trinnet med delvis denaturering af det genomiske DNA-molekyle omfattende målregionen ved hjælp af et af følgende trin:

i. ved hjælp af opvarmning af den eukaryotiske celle omfattende det genomiske DNA ved en temperatur i området 72 til 78 °C, fortrinsvis 75 °C, i 2 til 8 minutter, fortrinsvis 4 til 5 minutter, især 5 minutter,

ii. eller i nærvær af et kemisk middel, såsom formamid, f.eks. en opløsning af 70 % formamid,
eller ved hjælp af begge trin.

3. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 2, **kendetegnet ved, at** den første nukleotidsonde er et enkeltstrenget DNA-fragment, hvis størrelse varierer fra 80 bp til 3000 bp, eller fra 90 til 150 bp, især fra 95 til 110 bp, fortrinsvis med en størrelse på 99 bp.

4. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 3, **kendetegnet ved, at** den første nukleotidsonde er målrettet den mitokondrielle genomiske DNA-sekvens, der ligger mellem de to promotore PH1 (HSP) og LSP af det mitokondrielle genom af en eukaryotisk celle, eller er komplementær til en nukleinsyresekvens, der har mindst 80 % identitet med den mål-DNA-region, der ligger mellem de to promotore PH1 (HSP) og LSP af det mitokondrielle genom af en eukaryotisk celle.

5. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 4, **kendetegnet ved, at** den første nukleotidsonde, og eventuelt den anden nukleotidsonde, er direkte markeret med en fluorescerende gruppe og/eller omfatter modificerede nukleotider.

6. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 5, **kendetegnet ved, at** detekteringen af nukleinsyresekvensen af målregionen på det mitokondrielle DNA-genom og det mitokondrielle RNA-molekyle opnås i ét trin, især samtidigt.

7. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 6, **kendetegnet ved, at** den udføres på (en) fikseret/fikserede celle(r) eller væv.

8. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 7, til *in situ*-hybridisering og detektering af mitokondrielt nukleotidmateriale inden for mindst én eukaryotisk celle, omfattende trinnene:
a. At fiksere cellen i 1 til 4 % paraformaldehyd (PFA), fortrinsvis 2 % PFA i ca. 20 til 30 minutter, især 30 minutter,

b. At permeabilisere den fikserede celle med 0,5 % til 1 % Triton X100 i PBS 1X, i ca. 5 til 10 minutter ved 4 °C, især 5 minutter,
c. At denaturere nukleinsyreindholdet af den permeabiliserede fikserede celle ved hjælp af opvarmning ved en temperatur i et område på 72 til 78 °C, fortrinsvis 75 °C, i 2 til 8 minutter, fortrinsvis 4 til 5 minutter, især 5 minutter,
d. At bringe nukleinsyren/nukleinsyrerne i cellen behandlet i henhold til trin (c) i kontakt med mitokondriel(le) nukleotidsonde(r) til muliggørelse af hybridisering af mitokondriel(le) nukleinsyre(r) med sonden/sonderne, hvor sonden/sonderne har en størrelse i området fra 80 til 3000 nukleotider, eller fra 90 til 1000 nukleotider, især fra 95 til 110 nukleotider, hvilke(n) nukleotidsonde(r) er indeholdt i en hybridiseringsopløsning omfattende fra 100 ng/µl til 10 µg/µl laksesperm-DNA,
e. At detektere nukleinsyren/nukleinsyrerne hybridiseret til sonden/sonderne tilsat i trin (d).

9. Fremgangsmåde ifølge krav 8, yderligere omfattende et trin med at vaske den celle, der er bragt i kontakt med den/de mitokondrielle nukleotidsonde(r), med en egnet buffer inden detekteringstrinnet.

10. Fremgangsmåde ifølge et hvilket som helst af kravene 8 eller 9, hvor hybridiseringstrinnet udføres i løbet af ca. 15 timer ved 37 °C.

11. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 10, yderligere omfattende trin, der muliggør markering og detektering af mindst et protein af interesse i den eukaryotiske celle, hvor detekteringen af mitokondrielt nukleotidmateriale og af proteinet af interesse eventuelt opnås i ét trin, især samtidigt.

12. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 11, yderligere omfattende et trin med analyse af resultatet/resultaterne af den/de udførte detektering(er).

13. Nukleinsyremolekyle, der hybridiserer med en målregion i et eukaryotisk mitokondrielt genomisk DNA, hvor målregionen omfatter en nukleinsyresekvens, der ikke har nogen identificeret tilsvarende annealing RNA i den metabolisk aktive celle indeholdende det eukaryotiske mitokondrielle genomiske

DNA og derfor forbliver RNA-fri under transkription og replikation af det mitokondriele DNA-genom, hvor nukleinsyremolekylet er specifikt for et segment af ikke-transkriberet mitokondrielt gDNA, bestående af:

i. nukleinsyren med sekvensen med et hvilket som helst af SEQ ID N°1 til SEQ ID N°16 eller et komplement deraf eller

ii. en nukleinsyre, der har mindst 80 % identitet med nukleinsyresekvensen med et hvilket som helst af SEQ ID N°1 til SEQ ID N°16 eller et komplement deraf,

hvilket nukleinsyremolekyle er enten et enkeltstrenget molekyle eller et dobbeltstrenget molekyle.

14. Nukleinsyremolekyle ifølge krav 13, som er markeret.

15. Kit til udførelse af *in situ*-hybridisering på fikserede celler, omfattende en første nukleotidsonde bestående af et detekterbart nukleinsyremolekyle ifølge krav 13 eller krav 14 og omfattende en anden nukleotidsonde, der hybridiserer med et mitokondrielt RNA-molekyle.

16. Anvendelse af fremgangsmåden, nukleinsyremolekylet eller kittet ifølge et hvilket som helst af kravene 1 til 15 til detektering af mitokondriesygdom(me) eller til detektering af neoplastisk(e) sygdom(me) eller cancer(e) eller til test af cytotoksiciteten af organiske eller kemiske forbindelser, især lægemidler, på eukaryotiske celler.

DRAWINGS

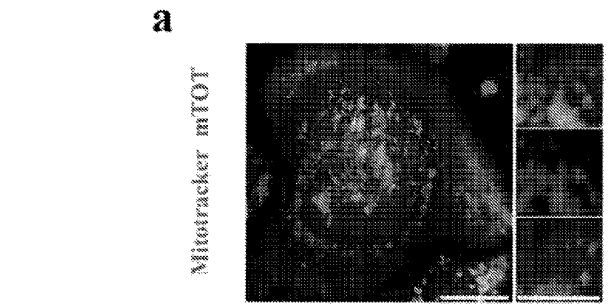

Fig. 1A

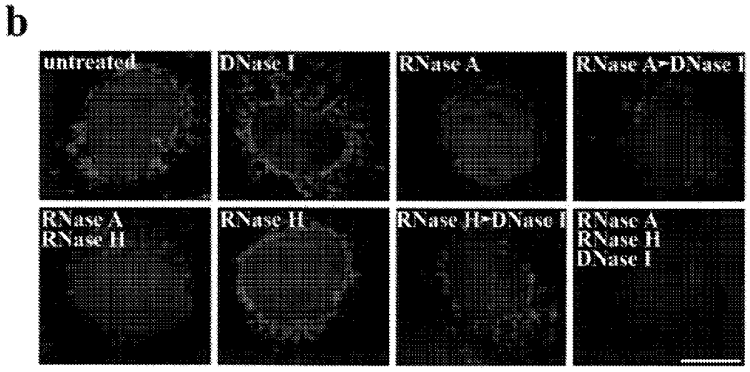

Fig. 1B

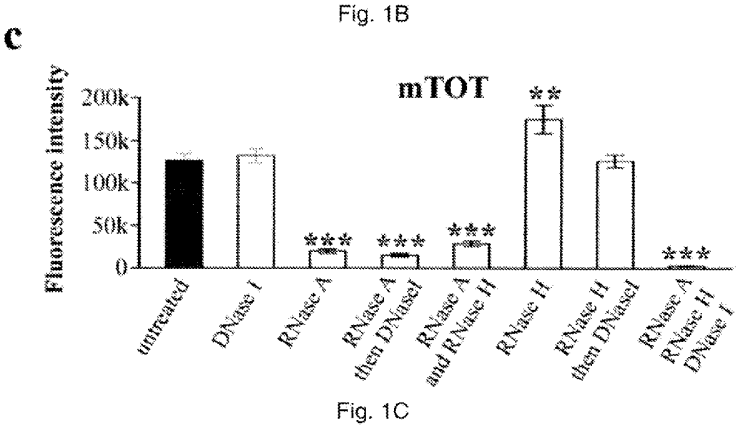

Fig. 1C

a

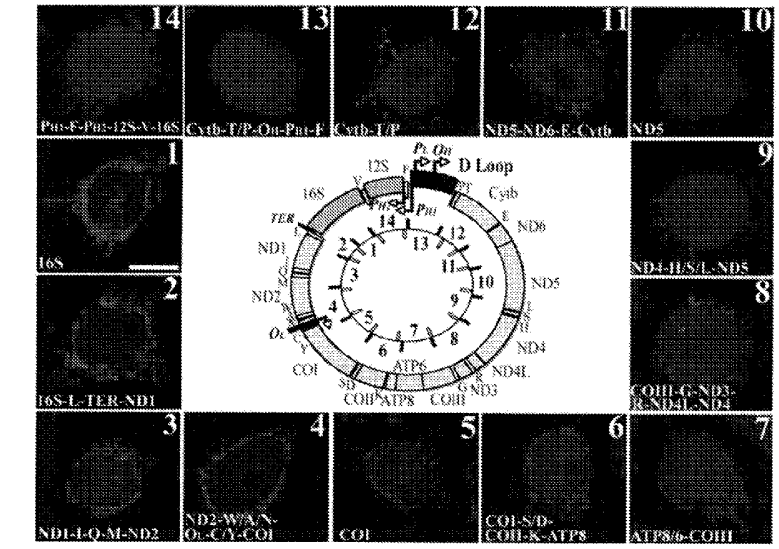

Fig. 2A

b

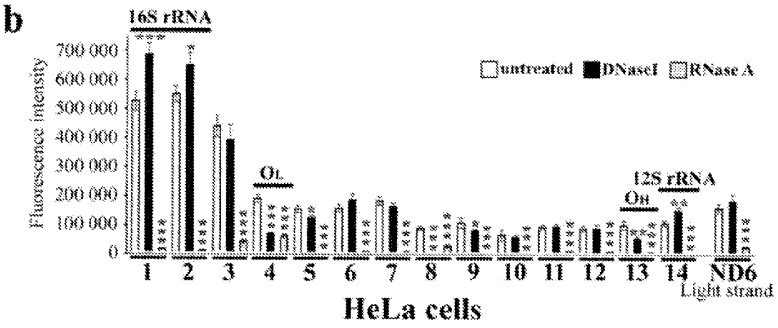

Fig. 2B

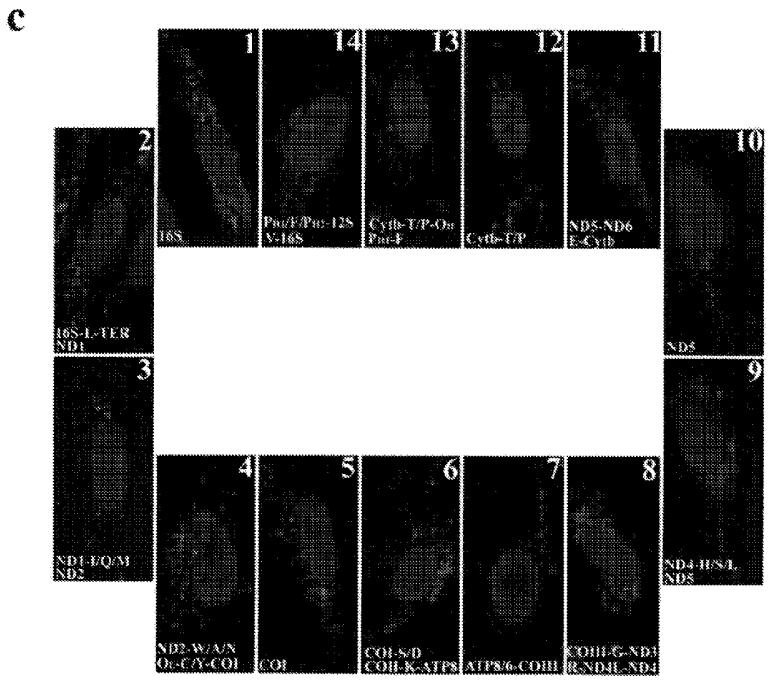
c
1
14
13
12
11
2
10
16S
Pro/F-Pro-12S V-16S
Cytb-T/P-Or Pro-F
Cytb-T/P
ND5-ND6 E-Cytb
16S-L-T/E/R ND1
ND5
3
9
ND1-I/Q/M ND2
ND4-H/S/L ND5
4
5
6
7
8
ND2-W/A/N Or-C/Y-COI
COI
COI-S/D COII-K-ATP8
ATP8/6-COIII
COIII-G-ND3 R-ND4L-ND4

Fig. 2C

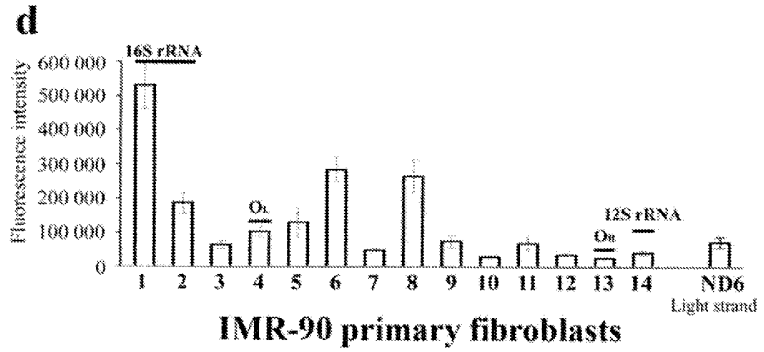
d
16S rRNA
600 000
500 000
400 000
300 000
200 000
100 000
0
Fluorescence intensity
Or
12S rRNA
Or
1 2 3 4 5 6 7 8 9 10 11 12 13 14
ND6
Light strand
IMR-90 primary fibroblasts

Fig. 2D

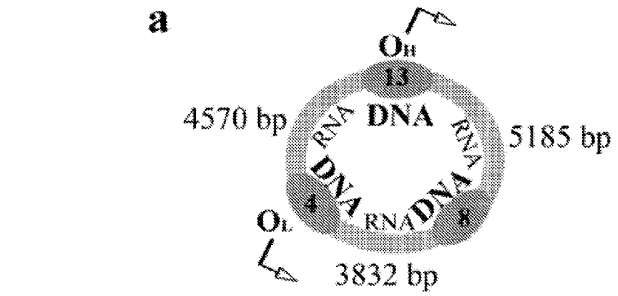

Fig. 3A

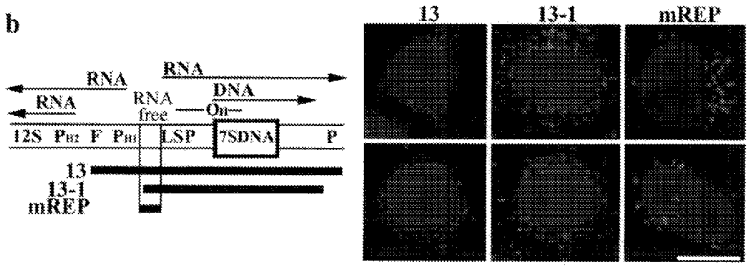

Fig. 3B

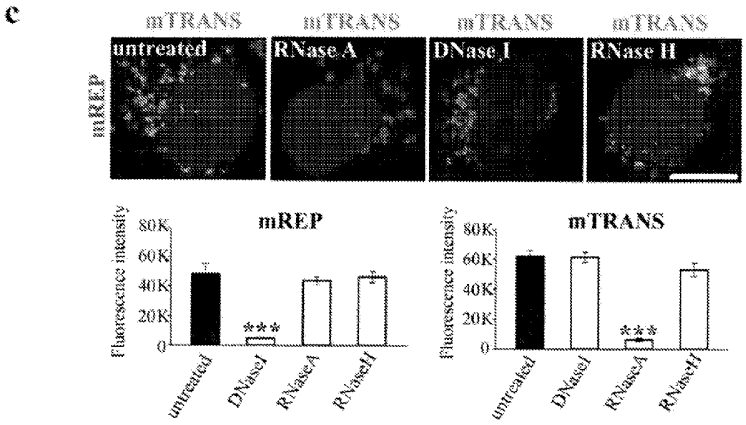

Fig. 3C

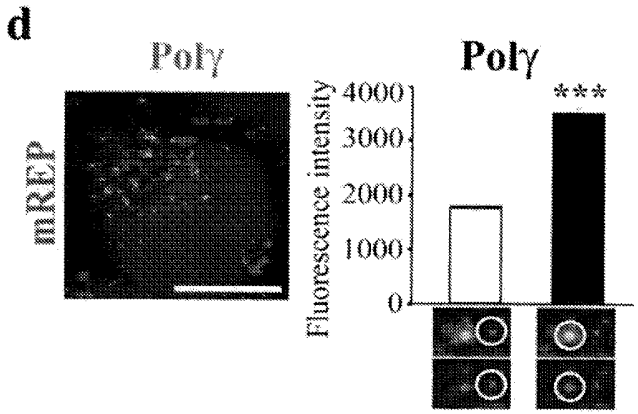
d
Polγ
mREP
Polγ
Fluorescence intensity
4000
3000
2000
1000
0
***

Fig. 3D

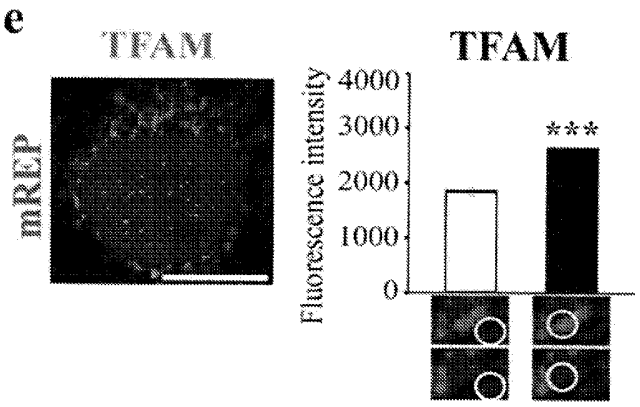
e
TFAM
mREP
TFAM
Fluorescence intensity
4000
3000
2000
1000
0
***

Fig. 3E

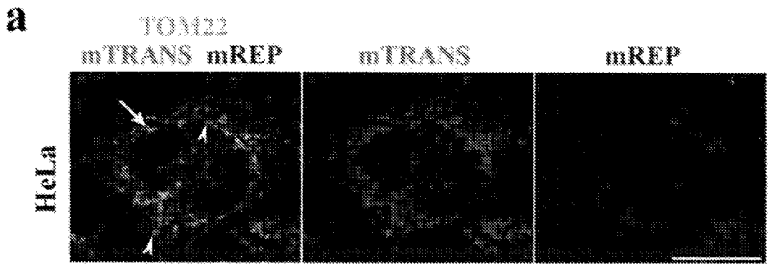

Fig. 4A

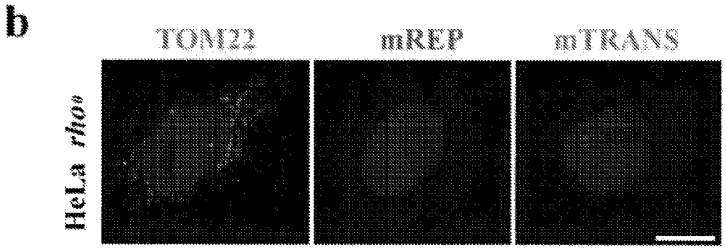

Fig. 4B

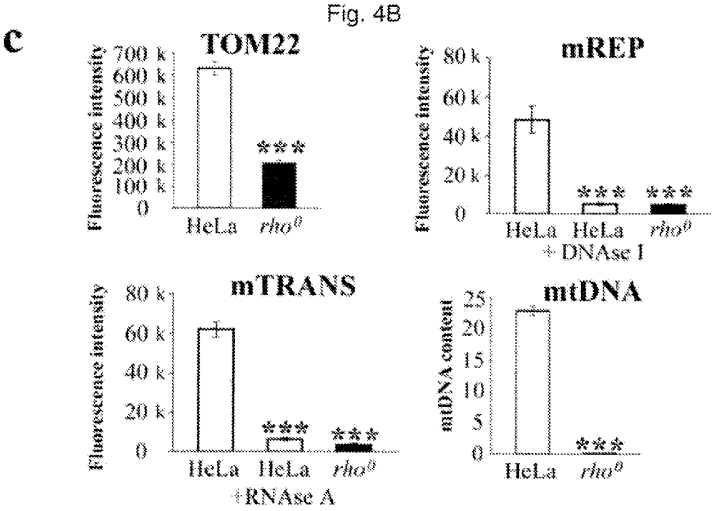

Fig. 4C

a

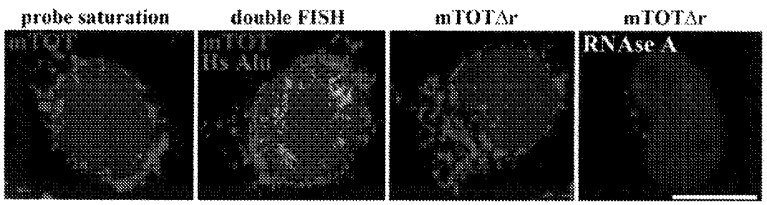

Fig. 5A

b

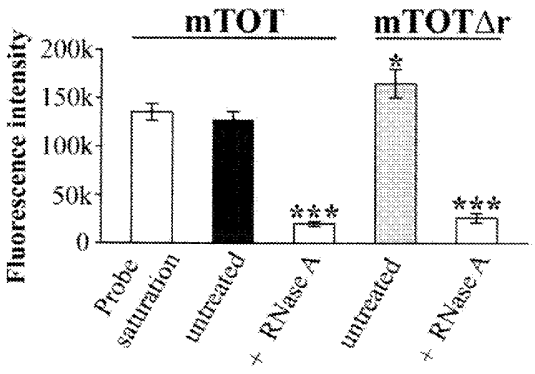

Fig. 5B

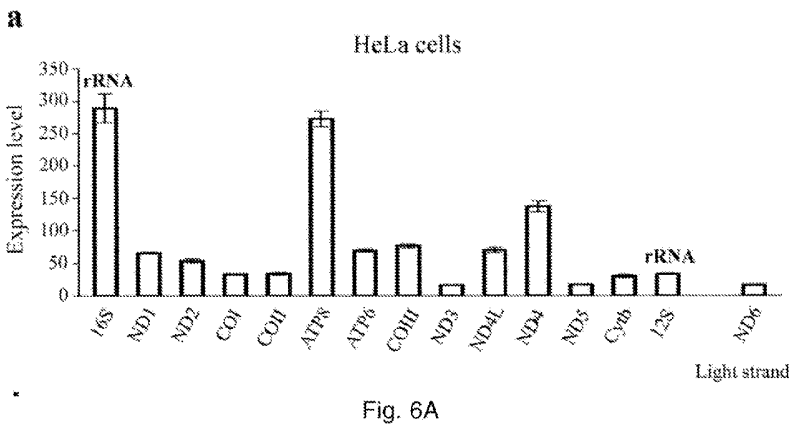
a
HeLa cells
Expression level
350
300
250
200
150
100
50
0
rRNA
rRNA
16S
ND1
ND2
CO1
COII
ATP8
ATP6
COIII
ND3
ND4L
ND4
ND5
Cytb
12S
ND6
Light strand

Fig. 6A

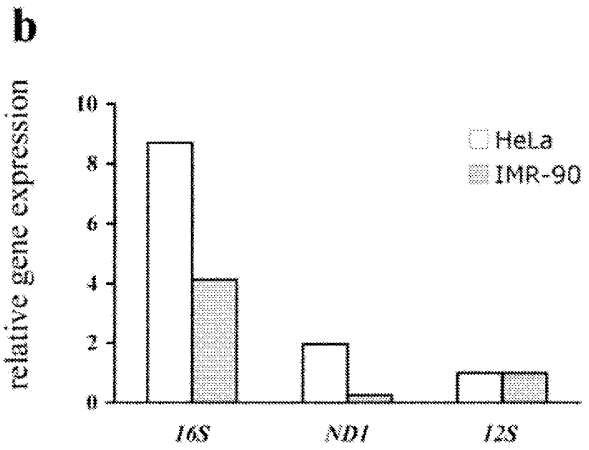
b
relative gene expression
10
8
6
4
2
0
HeLa
IMR-90
16S
ND1
12S

Fig. 6B

a

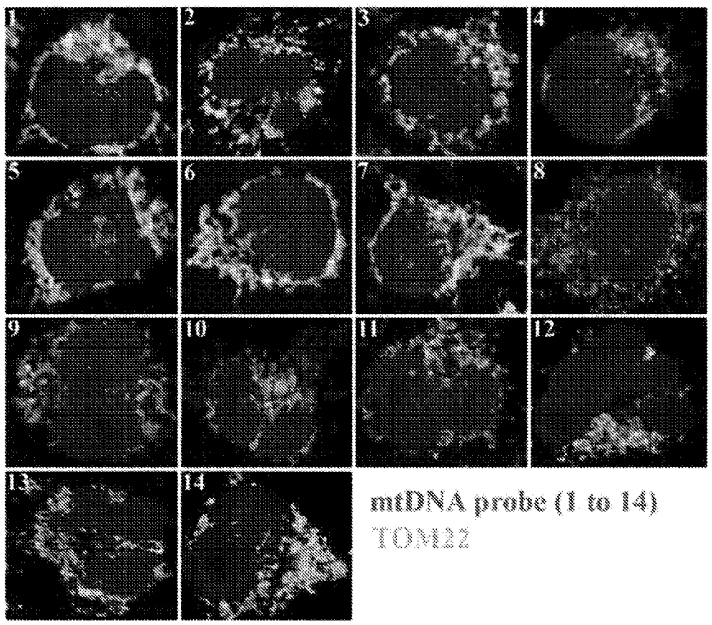

Fig. 7A

b

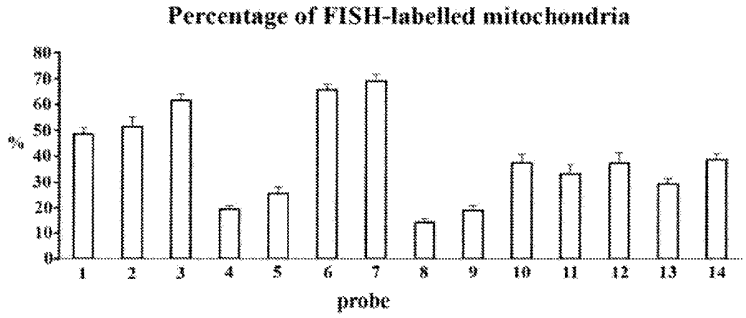

Fig. 7B

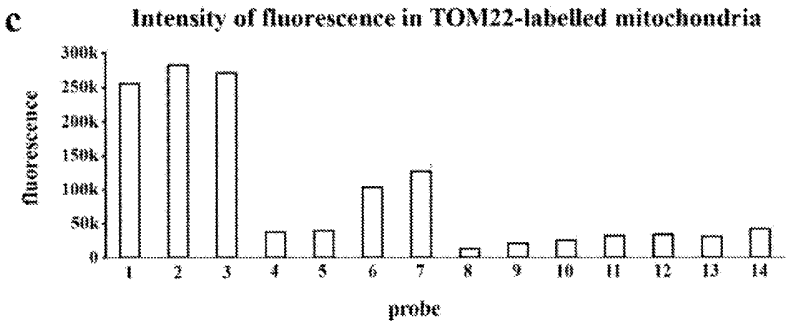
c
Intensity of fluorescence in TOM22-labelled mitochondria
fluorescence
300k
250k
200k
150k
100k
50k
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
probe

Fig. 7C

a

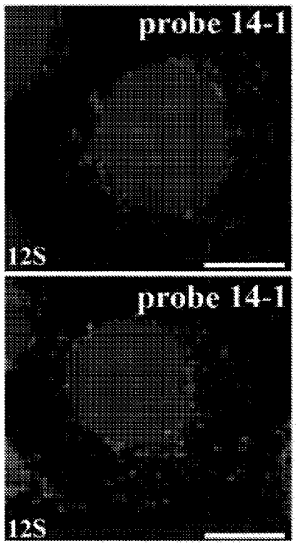

Fig. 8A

b

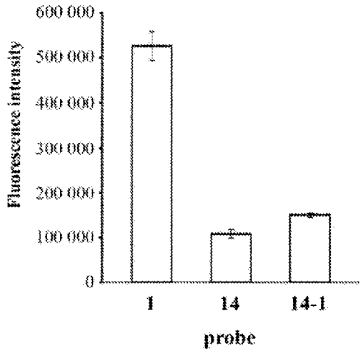

Fig. 8B

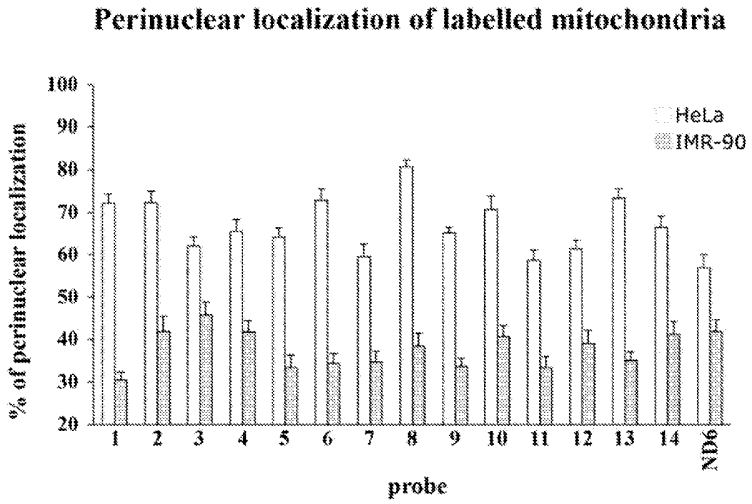
Perinuclear localization of labelled mitochondria
% of perinuclear localization
100
90
80
70
60
50
40
30
20
HeLa
IMR-90
1
2
3
4
5
6
7
8
9
10
11
12
13
14
ND6
probe

Fig. 9

a

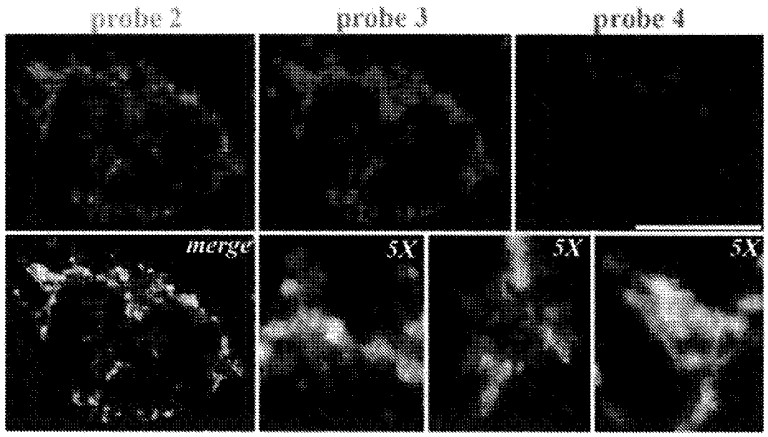

Fig. 10A

b

Probe	% of co-localization with 2	3	4
2	-	92.1 ± 1.4	22.6 ± 3.2
3	84.3 ± 1.9	-	26.8 ± 3.2
4	44.8 ± 3.0	50.5 ± 4.2	-

Fig. 10B

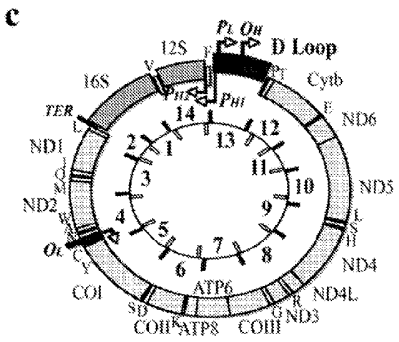

Fig. 10C

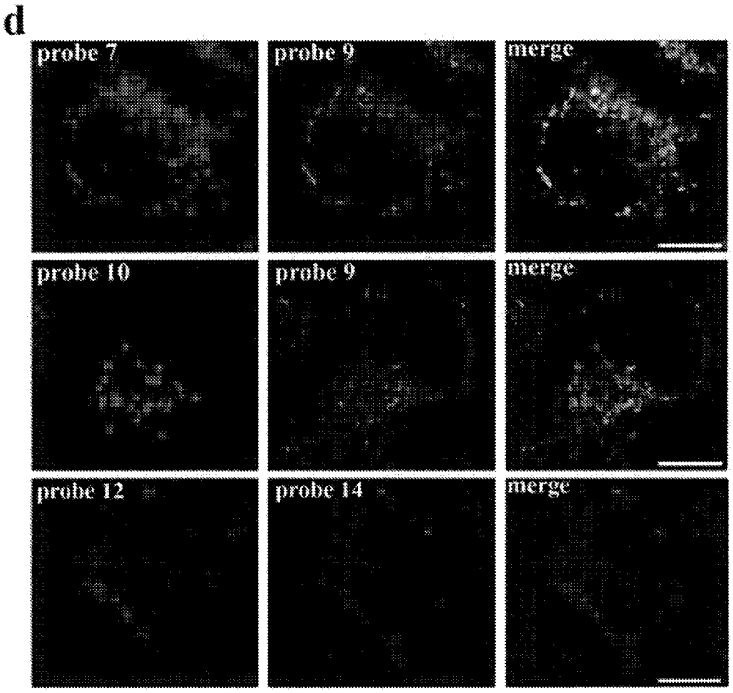

Fig. 10D

e

probe	% of co-localization with 7	9
7	-	59.4 ± 1.4
9	98.7 ± 0.3	-

probe	9	10
9	-	57.6 ± 4.7
10	40.3 ± 2.5	-

probe	12	14
12	-	28.2 ± 2.0
14	55.6 ± 7.0	-

Fig. 10E

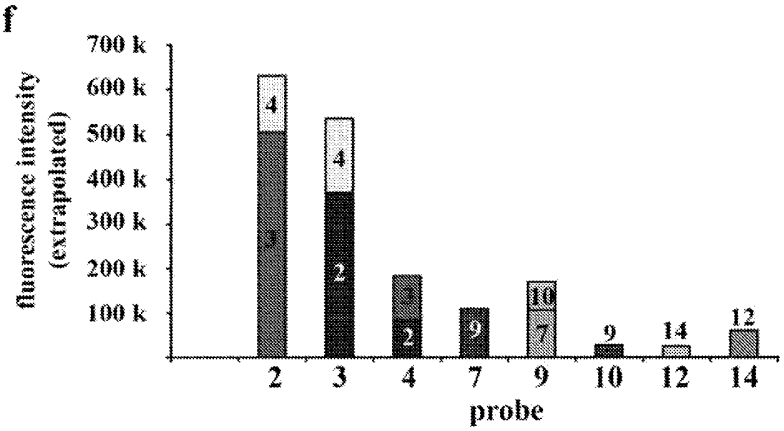

Fig. 10F

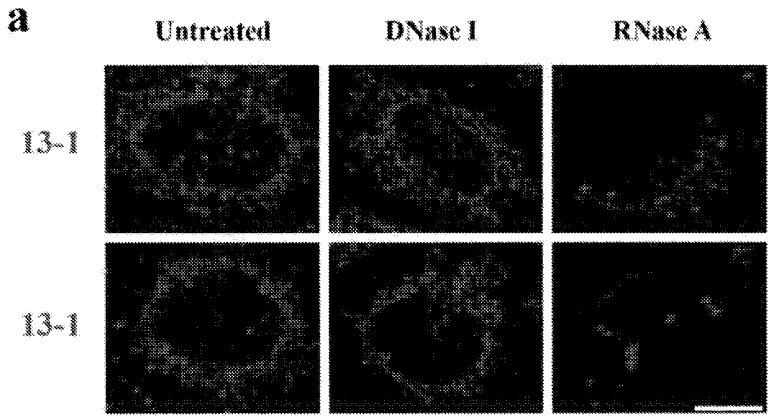

Fig. 11A

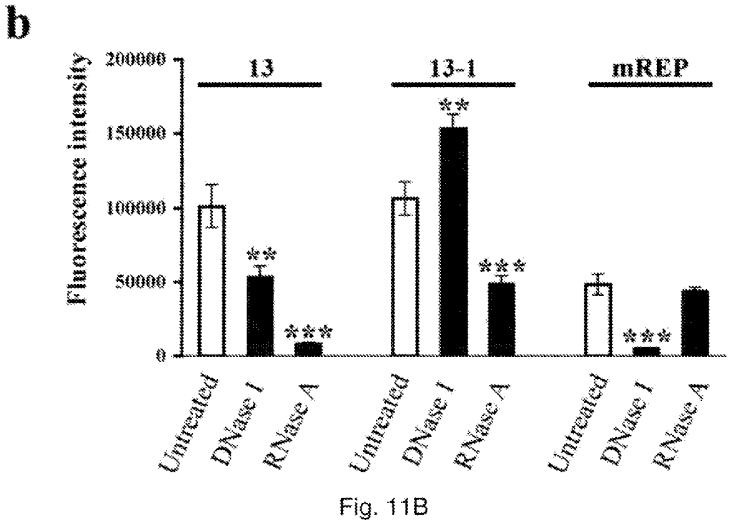

Fig. 11B

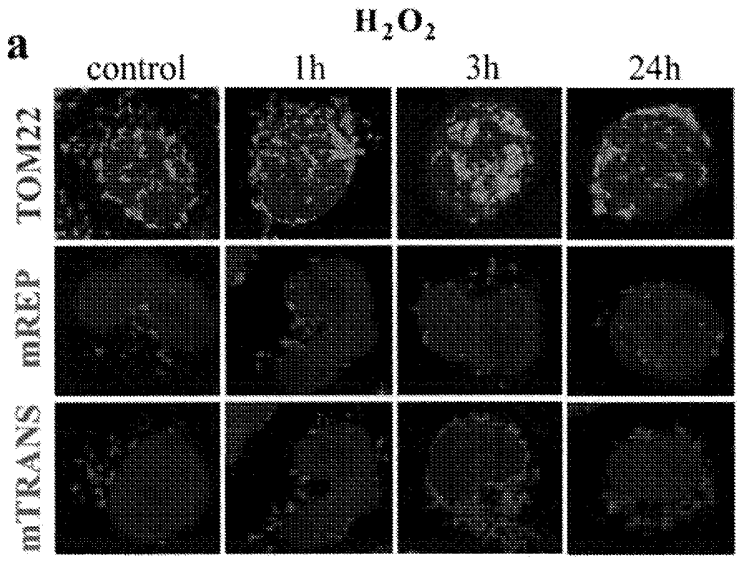

Fig. 12A

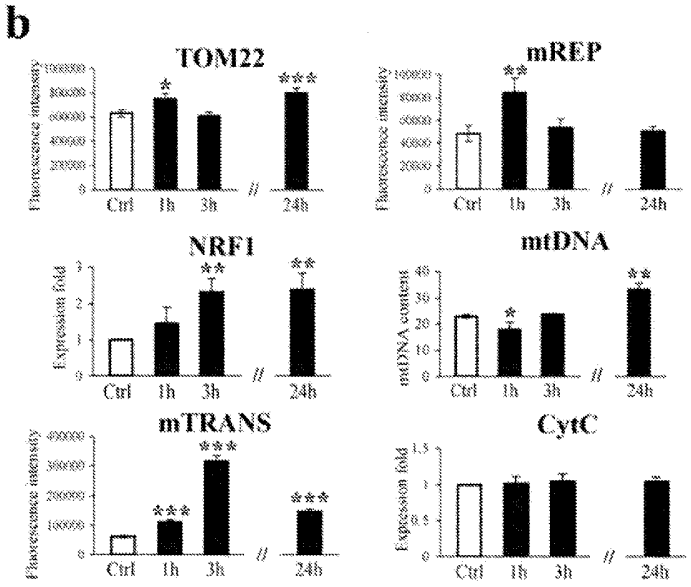
b
TOM22
mREP
NRF1
mtDNA
mTRANS
CytC
Fluorescence intensity
Expression fold
mtDNA content
Ctrl
1h
3h
24h

Fig. 12B

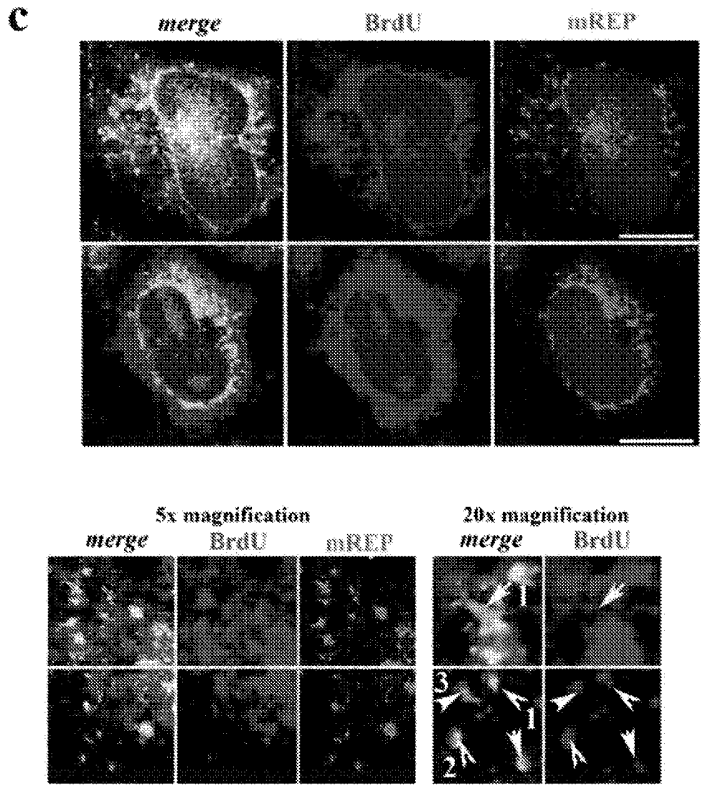
C
merge
BrdU
mREP
5x magnification
merge
BrdU
mREP
20x magnification
merge
BrdU
1
3
1
2

Fig. 12C

Human mREP sequence (99)

```
ACATTATTTTCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCCGCCCATCCTAC
CCAGCACACACACACCGCTGCTAACCCCATACCCC
```

Fig. 13A

mREP - Human polymorphism – variations

Fig. 13B

-> Homo sapiens NC_012920.1

```
HsmREP  ACATTATTTTCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCCGCCCATC
Hs      ACATTATTTTCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCCGCCCATC
        ************************************************************

HsmREP  CTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Hs      CTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
        ****************************************
```

-> Pan troglodytes NC_001643.1

```
HsmREP  ACATTATTTTCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCC-GCCCAT
Pt      ACATGCCCTCCCCCCTCAACTCCCATTCTACTAGCCCCAGCAACGTAACCCCCTACTCAC
        ****    * ****  * ******** ******  * ** ***   *******  * **

HsmREP  CCTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Pt      CCTACTCA--ACACATATACCGCTGCTAACCCCATACCCT
        ***** **  ***** * **********************
```

-> Pan paniscus NC_001644.1

```
HsmREP  ACATTATTTTCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCC-GCCCAT
Panp    ACATGTCCTCCCCCCTCAACTCCCATTCCACTAGCCCCAACAACATAACCCCCTGCCCAC
        ****    * ****  * ******** * ****  * ** *** * ******* *****

HsmREP  CCTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Panp    CCCACTCAGCACATATAC--CGCTGCTAACCCTATACCCT
        ** ** ******* * **  ************ ******
```

Fig. 13C.1

-> Gorilla gorilla NC_001645.1

```
HsmREP  ACAT-TATTTTCCCCTCCCACTCCCATACTACTA-----ATCTCATCAATACAACCCCCG
Gg      GTATGCACTTTTAACAGTCACCCCTCAACTAACATAGTCAGCCCACCAGTACAACCCCCG
          **  * ***   *   *** **   **** *      * * ** ** **********

HsmREP  CCCATCCTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Gg      CCCGCCCTA----GCA-ACACACACTGCTGCTGATCCTATACCCC
         ***  ****    *** ******** ****** * ** *******
```

-> Pongo pygmaeus NC_001646.1

```
HsmREP  ACAT-TATTTTCCCCTCCCACTCCCATACTACTA-ATCTCATCAATA----CAACC--CC
Pp      ATGTGCACTTTCAACAGGCACCCCTCAACTAACACAACCCACTTTTAATTTCCACCTACC
        *  *  * ****  *   *** **   ****  * * * **   **    * ***  **

HsmREP  CGCCCATCCTACCCAGCA----CACACACACCGCTGCTAACCCCATACCCC
Pp      AACCCATCCTGCCCTGCCTTCCCACAAACACCACTACTACCCCCACACCTC
          ******** *** **     **** ***** ** *** ***** *** *
```

-> Hylobates lar NC_002082.1

```
HsmREP  ACATT--ATTTTCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCCGCCCA
Hl      ACATTCCATATTCCAGCCGAGCATCCAATCCACTAAAGGTGCTAAT-TAATTCATGCTTG
        *****  ** ****   *   *  ***  * *****       ***  **  *  **

HsmREP  TCCTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Hl      TTGGACATAGCAATAACCAACCAACG-TAACCCCAAACCAC
        *   **  ****   **  ***   * ******** *** *
```

-> Cebus albifrons NC_002763.1

```
HsmREP  ACATTATTTTCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCCGCCCATC
Ca      GCATTTGGTTCCTACCTCAGGGCCATCTCACTAAGACCGTGTCCACGTTCCTCTTAAATA
         ****   ****   *  *   ****   *****   * *    **   ** *    **

HsmREP  CTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Ca      AGACATCACGATGGTGTGGCGCTATCACCCTCTTAACCG
          **    *          ****   * ** * ** **
```

-> Capra hircus NC_005044.2

```
HsmREP  ACATTATTTTCCCCTCCCAC-----TCC---CATACTACTAATCT-----CATCAATACA
Ch      ACACAAACTTCCCACTCCACAAGCTTACAGACATGCCAACAACCCACACGTATAAAAACA
        ***  *  *****   ****      * *   *** * *  ** *       *  ** ***

HsmREP  ACCCCCGCCCATCCTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Ch      TCCCAATCCTAACCCAACTTAGATACCCACACAAACGCCAACACCACACAAT
         ***   ** * ** * *    * ** *****    ** *** *** **
```

Fig. 13C.2

-> Mus musculus NC_005089.1

```
HsmREP  ACATTATTTTCCCCTCCCACTCCCATACTACTAATCT-----CATCAATACAACCCCCGC
Mm      ATATGACTATCCCCTTCCCCATTTGGTCTATTAATCTACCATCCTCCGTGAAACCAACAA
        * ** * * ****** ** *         *** ******     * **  *  ****  *

HsmREP  CCATCCTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Mm      CCCGCCCACCAATGCCCCTCTTCTCGGTCCGGGCCCATTAAACT
        **  ** *** *   * * *    **** *   ***  **  *
```

-> Oryctolagus cuniculus NC_001913.1

```
HsmREP  ACATTATTT--TCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCCGCCCA
Oc      AGACCATCAAATCTACACACACCACTCAACTCTTACCCATACGACTATCCCTCTCCCCCA
        * *  **    **  * * ***  *   ***  **  *  *  * **   * * * ****

HsmREP  TCCTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Oc      GTCCTCTCACAACTTACCATCCTCCGTGAAACCAACAACCC
          *  * **  **  **   ** * *  ** ** * * ***
```

-> Canis lupus NC_008092.1

```
HsmREP  ACAT-TATTTTCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCCGCCCAT
Cl      ACTTATACAAACCCCCCTTACCCCCCCGTAAACTCATGTCATCTATTATACACTTATTTAT
        ** * **    **** *  ** ***      *** ** ***** **   ** *       **

HsmREP  CCTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Cl      GTCCCGCCAAACCCCAAAAACAGGACTAAGTGCATACAAT
          * *   ** *  * * *    ****   *****
```

-> Rattus sordidus NC_014871.1

```
HsmREP  ACATTA-TTTTCCCCTCCCACTCCCATACTACTAATCTCA-TCAATACAACCCCCGCCCA
Rs      TCATAAACCTTTCTCTTCCATATGACTATCCCTGACCCCAATTGGTCTATATTTCTACCA
         *** *   ** * ** ***       **   ** * * ** *   *  *     *  ***

HsmREP  TCCTACCCAGCACACACACACCGCT-GCTAACCCCATACCCC
Rs      TCCTCCGTGAAATCAACAACCCGCCCACTAGTCCCCCTCTCC
        **** *     *   ***  ****   ***  ***   * **
```

-> Felis catus NC_001700.1

```
HsmREP  ACATTATTTTCCCCTCCCACTCCCA--TACTACTAATCTCATCAATACAACCCCCGCCCA
Fc      ATACTAAATCATAACTCTGTTCGCAGTTATCTATAGATATACCGACCTGACTCTAATTCG
        * * **  *        *   ** **  **     **      *   *    ** *     *

HsmREP  TCC-TACCCAGCA----CACACACACCGCTGCTAACCCCATACCCC
Fc      TCCCTATCGAACAACATTTTACATGTCTACGTTAGCCCCACATCCC
        *** ** * * **       ***   *   * ** ***** * ***
```

Fig. 13C.3

-> Castor canadensis NC_015108.1

```
HsmREP  ACATTATTTTCCCCTCCCACTC-CCATACTACTAAT--CTCATCAATACAACCCCCGCCC
Cc      ACAGTCTCTTAATCTACCATCCTCCGTGAAACCAGCAACCCGCTCGGGGAATGTCCCCTC
        *** * * **    ** ***  * ** *   ** *     * *          **   ** * *

HsmREP  ATCCTACCCAGCACACACACACCGCTGCTAACCCCATACCCC
Cc      TTCTCGCTCCGGGCCCATACAACTTGIGGGTTTCTATTCTGA
         **   * * *  * ** *** *   *       * ** *
```

-> Gallus gallus NC_001323.1

```
HsmREP  ACATTATTTTCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCCGCCCATC
Ggal    CCATTCTTTCCCCCTACACCCCTCGCCCTACTTGCCT--TCCACCGTACCTCTGGTTCCT
         **** *** ***** *  * * *   *****   **  ** *    *** * *

HsmREP  CTACCCAGCACA---CACACACCGCTGCTAACCCCATACCCC
Ggal    CGGTCAGGCACATCCCATGCATAACTCCTGAACTTTCTCACT
        *   *  *****   **  **   ** ** * *     * *
```

-> Danio rerio NC_002333.2

```
HsmREP  ACATTATTTTCCCCTCCCACTCCCATACTACTAATCTCATCAATACAACCCCCGCCCATC
Dr      ACTATATATTATTATCTCCCCCTTTTGGTA-TACGCGCGACAA-ACCCCCTTACCCCCTT
        **  *** **    ** * * *   *  ** **  * *  *** **  **    *** *

HsmREP  CTACCCAGCACACACA--CACCGCTGCTAACCCCATACCCC
Dr      ACGTCCAGCGATTCCTGTTATCCTTGTCAAACCCCTAAACC
            *****     *   * *  **  ** **  **  **
```

Fig. 13C.4

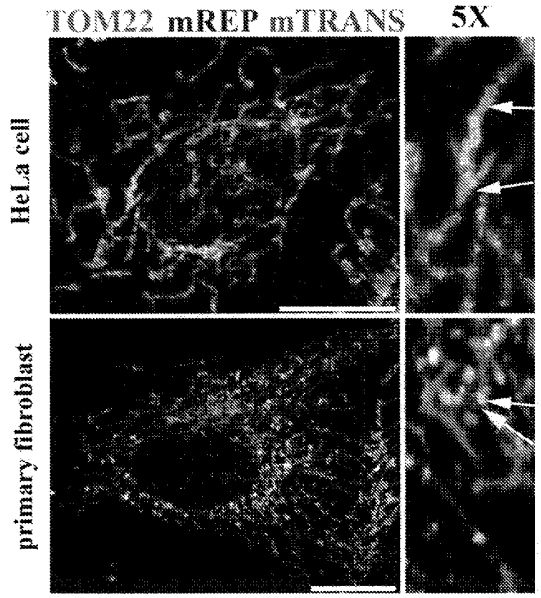

Fig. 14A

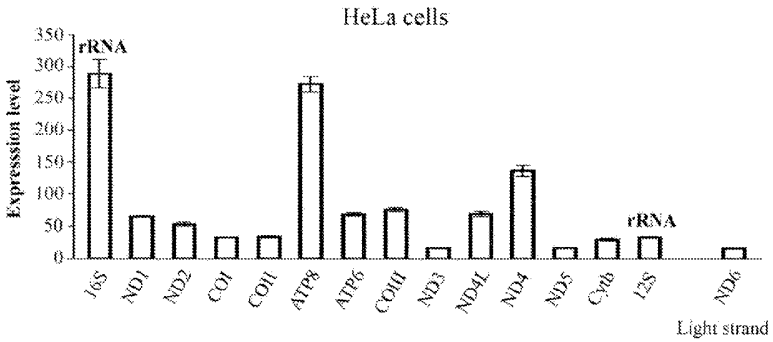

Fig. 14B

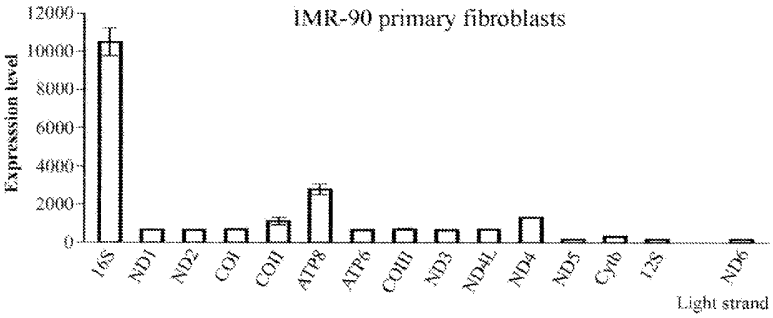

Fig. 14C

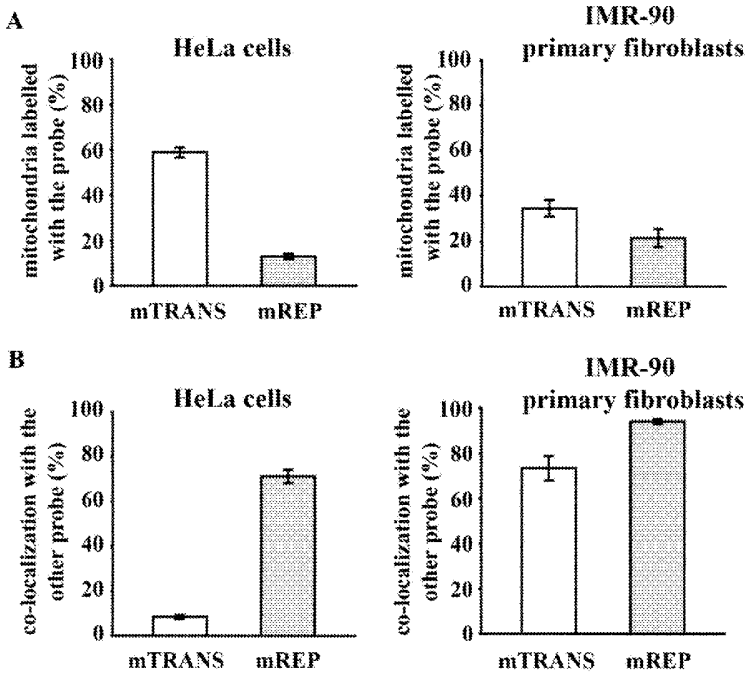
A
HeLa cells
IMR-90
primary fibroblasts
mitochondria labelled with the probe (%)
100
80
60
40
20
0
mTRANS
mREP
B
HeLa cells
IMR-90
primary fibroblasts
co-localization with the other probe (%)
100
80
60
40
20
0
mTRANS
mREP

Fig. 14D