Chapter 5

A Single-Cell Resolution Imaging Protocol of Mitochondrial DNA Dynamics in Physiopathology, mTRIP, Which Also Evaluates Sublethal Cytotoxicity

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Abstract

Mitochondria autonomously replicate and transcribe their own genome, which is present in multiple copies in the organelle. Transcription and replication of the mitochondrial DNA (mtDNA), which are defined here as mtDNA processing, are essential for mitochondrial function. The extent, efficiency, and coordination of mtDNA processing are key parameters of the mitochondrial state in living cells. Recently, single-cell analysis of mtDNA processing revealed a large and dynamic heterogeneity of mitochondrial populations in single cells, which is linked to mitochondrial function and is altered during disease. This was achieved using *m*itochondrial *T*ranscription and *R*eplication *I*maging *P*rotocol (mTRIP), a modified fluorescence in situ hybridization (FISH) approach that simultaneously reveals the mitochondrial RNA content and mtDNA engaged in initiation of replication at the single-cell level. mTRIP can also be coupled to immunofluorescence or MitoTracker, resulting in the additional labeling of proteins or active mitochondria, respectively. Therefore, mTRIP detects quantitative and qualitative alterations of the dynamics of mtDNA processing in human cells that respond to physiological changes or result from diseases. In addition, we show here that mTRIP is a rather sensitive tool for detecting mitochondrial alterations that may lead to loss of cell viability, and is thereby a useful tool for monitoring sublethal cytotoxicity for instance during chronic drug treatment.

Key words Mitochondrial DNA, FISH, Imaging, Metabolism, Transcription, DNA replication, Single-cell, Cytotoxicity, Long-term drug treatment

1 Introduction

Mitochondria play a central role in cellular metabolism and are also involved in cell signaling and cell death. Mitochondria depend on the nuclear genome and on their own genome, thereby requiring continuous and efficient nuclear-mitochondrial communication. This is the case for mitochondrial DNA (mtDNA) replication and transcription, which take place in the organelle, but all mtDNA processing factors are coded in the nucleus [1, 2]. The mitochondrial genome, a double-stranded circular DNA of 16.6 kbp in humans, is present in many copies *per* cell and is organized in

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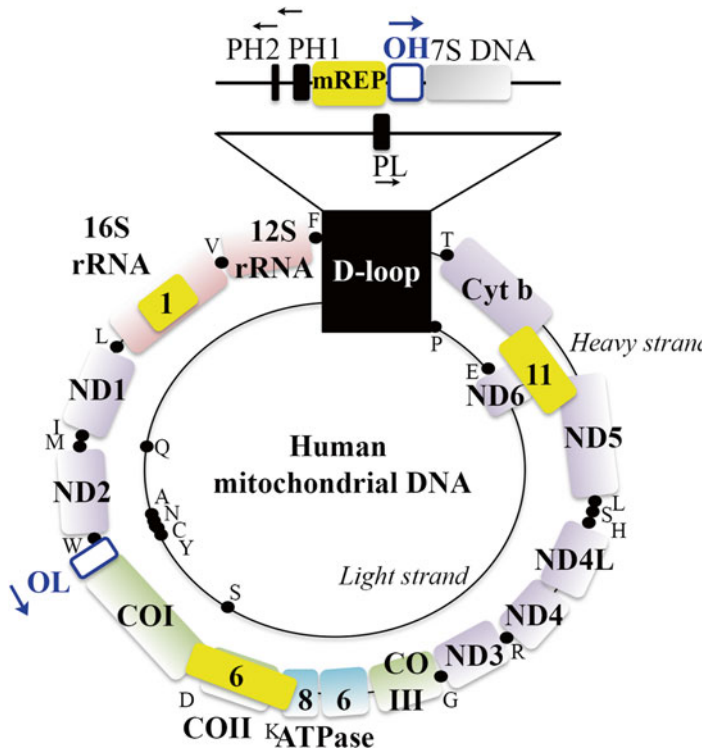

Fig. 1 Human mitochondrial DNA and probes used in mTRIP. Both the H (heavy) and light (L) strands of the mitochondrial genome are indicated (external and inner circle, respectively). The position and name of individual genes are also represented (not at scale), and tRNAs are indicated with a *black dot* and identified with the *corresponding letter*. With the exception of *ND6* and several tRNAs located on the L-strand, all genes are located on the H-strand. The position of probes 1, 6, and 11, which cumulatively constitute the probe mTRANS, is indicated with a *yellow box*. mTRANS targets rRNA as well as several coding genes and tRNA on both strands. Any given mitochondrial transcript or fraction of can be visualized by mTRIP, using the specific probe. The D-loop region, which contains the origin of replication of the H-strand (O_H) and promoters of both H (PH1 and PH2) and L (PL) strands, is zoomed in on top of the scheme; within this region is also shown the position of the mREP probe. The direction of transcription is indicated with a *black arrow* at the level of each promoter; the direction of replication is indicated with a *blue arrow*

multiple protein-containing nucleoids *per* mitochondrion. The human mtDNA codes for two ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs), and 13 proteins that function in the respiratory chain (Fig. 1). Mitochondrial RNA is transcribed into polycistronic precursor RNAs and then processed to mature mRNAs, rRNAs, and tRNAs [3]. Therefore, processed and unprocessed RNA molecules may coexist in mitochondria. Distinct models are proposed

for replication of mtDNA [4–7], but it is accepted that replication starts at the O_H origin in the non-coding regulatory region. This generates full-length DNA and also a short molecule, the 7S DNA, that forms a triple-stranded region, the D-loop. The function of 7S has not been elucidated [8].

Mitochondrial replication and transcription have for long time been considered independent from nuclear replication, but recent evidences support a coordination of mtDNA processing with the cell cycle [9–11]. MtDNA replication seems to be heterogeneous among mitochondria and nucleoids. Indeed, super-resolution imaging revealed that mtDNA replication occurs only in a subset of nucleoids [12, 13]. Moreover, we showed a dramatic heterogeneity in mtDNA transcription and initiation of replication in single cells [14], suggesting that the dynamics of mtDNA processing are distinct according to cell type, growth condition, and metabolic state. In this context, the extent and coordination of mtDNA processing may act as markers of changing mitochondrial states and also disease [9, 14].

The dynamics of mtDNA processing can be assessed using mTRIP, an imaging protocol based on modified FISH, that simultaneously detects and quantifies the mitochondrial RNA (mtRNA) content and mtDNA engaged in initiation of replication in single cells [14] (Figs. 2 and 3). Since mTRIP can be coupled to immunofluorescence, proteins are also visualized and their signal quantified. Based on a milder treatment than traditional FISH, mTRIP generally does not damage protein epitopes (or does so mildly), thereby allowing the simultaneous tracking of mitochondrial nucleic acids (DNA and RNA) and proteins.

mTRIP is generally performed using mREP and mTRANS probes. The mREP probe specifically detects mtDNA engaged in initiation of replication through specific binding to a DNA region located upstream of the replication origin O_H in the D-loop (Fig. 1). mREP specifically recognizes target DNA in an open structure conformation, therefore it does not bind to double-stranded mitochondrial genomes that are replication-inactive. The sequence and size of this probe are unique, therefore mREP cannot be substituted with another probe for labeling initiation of mtDNA replication. mREP labeling does not differentiate whether initiation of replication will led to full-length DNA or short 7S DNA.

mTRANS, which detects selected mtRNAs considered to be representative of global mitochondrial transcripts, is a mix of three DNA probes that recognize distinct regions of the mitochondrial genome (Fig. 1). These regions are devoid of major replication origins, include a combination of rRNA, tRNAs, and mRNAs coded on both strands, and are located at regular distances on the mitochondrial genome. Differently from mREP, the mTRANS mix is not unique and any transcript of interest can be specifically

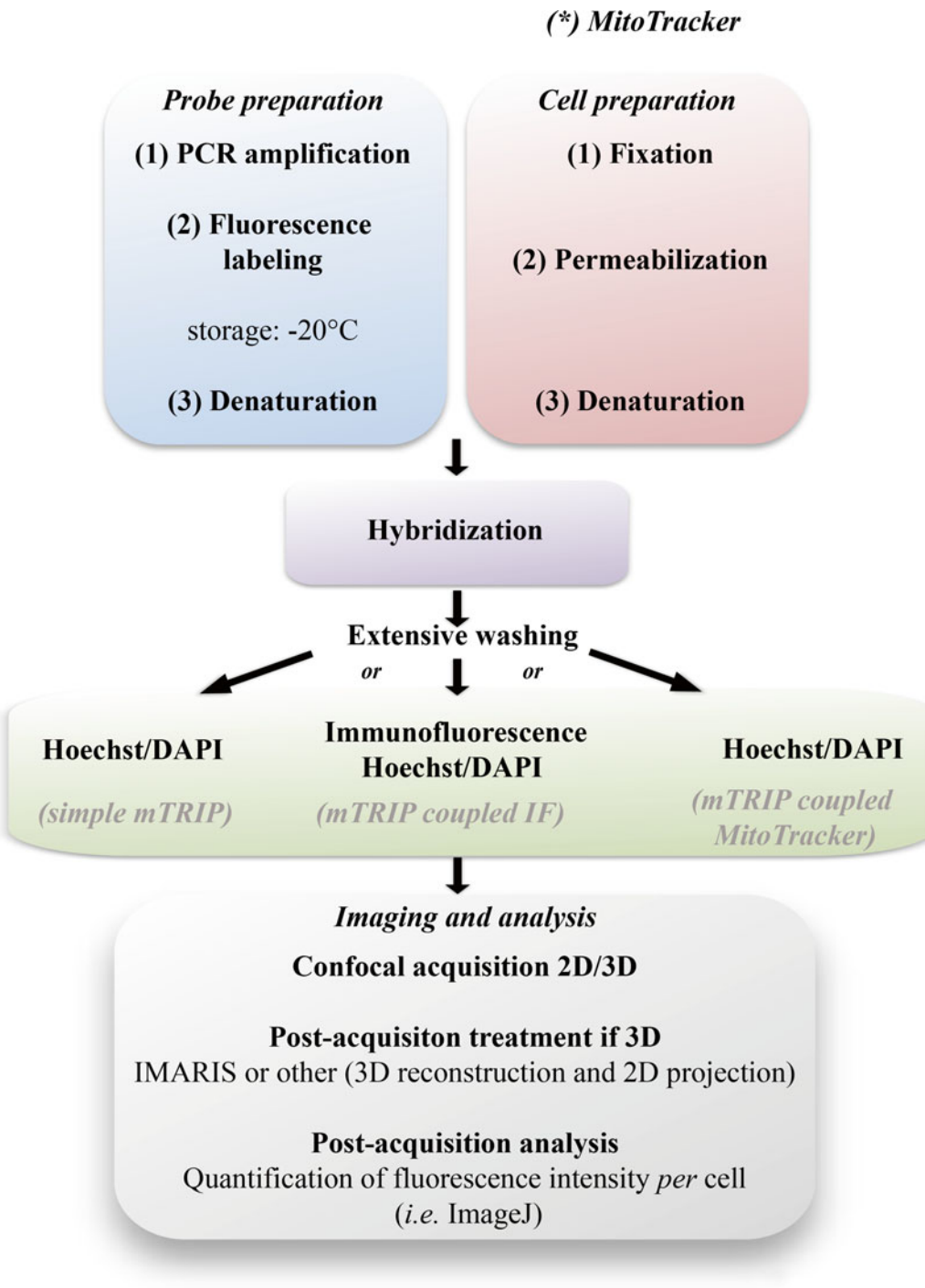

Fig. 2 Scheme of the mTRIP protocol and possible couplings. The key steps of mTRIP labeling are indicated, with preparation of probes and cells shown in parallel. Note that if the MitoTracker labeling is planned (indicated with an *asterisk* in the schema), the compound should be added to the cell culture for the required time, before cell fixation. The following steps until hybridization and washing are common to all procedures. After hybridization and washing, if the mTRIP labeling is done alone (or cells have been previously labeled with MitoTracker), cells must just be stained with Hoechst/DAPI to mark the nucleus. Alternatively, if mTRIP is coupled to immunofluorescence, immunostaining should be performed at this stage, followed by Hoechst/DAPI staining to mark the nucleus. The imaging protocol is common to all procedures; 3D acquisition allows fluorescence quantification of the entire cell volume, but 2D acquisition (and fluorescence quantification) of surfaces on individual sections can also be performed

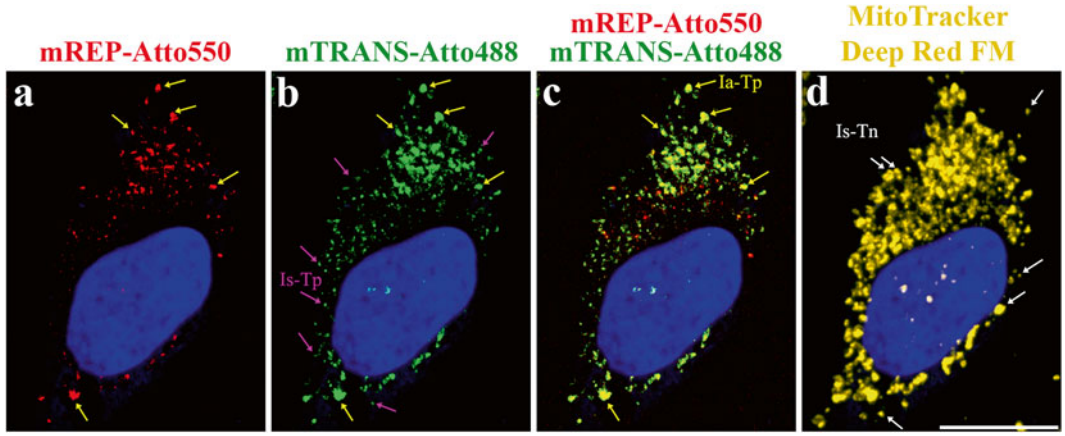

Fig. 3 mTRIP coupled to MitoTracker® Deep Red FM. mTRIP labeling of a human HeLa cell with (**a**) mREP (*red*) and (**b**) mTRANS (*green*) shows mitochondrial DNA initiation of replication and global mitochondrial transcripts, respectively. (**c**) Co-labeling with mREP and mTRANS. (**d**) The mitochondrial network is labeled by MitoTracker Deep® Red FM (*yellow*). While mREP essentially colocalizes with mTRANS (a few $mREP^{+}/mTRANS^{+}$ structures (or Ia-Tp) are indicated with *yellow arrows* in panels **a**, **b**, **c**), independent mTRANS labeling without colocalization with mREP (a few $mREP^{-}/mTRANS^{+}$ structures (or Is-Tp) are indicated with *purple arrows* in panel **b**), as well as mitochondria with undetectable levels of mREP and mTRANS (a few $mREP^{-}/mTRANS^{-}$ structures (or Is-Tn) are indicated with *white arrows* in panels **d**) are also detected. Ia-Tp = Initiation of replication-active and transcription-positive; Is-Tp = Initiation of replication-silent and transcription-positive; Is-Tn = Initiation of replication-silent and transcription-negative. Scale bar = 10 μm

targeted through the design of specific probes (*see* Ref. 14). mTRANS, and any other probe that identifies mtRNAs, will label the target transcripts in the processed as well as unprocessed form.

In a development of this approach, the mitochondrial network can also be tracked. For this, we combined mTRIP with either MitoTracker [15] or immunostaining of a mitochondrial outer membrane protein (TOM22 or TOMM22) [14]. This labeling allows the detection and quantification of at least three classes of mitochondrial subpopulations: (1) replication initiation active and transcript-positive (Ia-Tp); (2) replication initiation silent and transcript-positive (Is-Tp); and (3) replication initiation silent and transcript-negative (Is-Tn) [14]. These mitochondrial subpopulations are highly dynamic and we showed that they vary in number during the cell cycle, under stress, and disease [9, 14]. Thus, isolated or combined mREP and mTRIP labeling (either coupled with labeling of the mitochondrial network or of proteins of interest) act as indicators of the mitochondrial state.

Given the relevance of mtDNA maintenance and expression for the cell function, mTRIP markers may also reveal mitochondrial impairment, eventually associated with cytotoxicity, at an early stage. In Fig. 4 we show that individual mREP and mTRANS labeling detect alteration of mitochondrial parameters at low

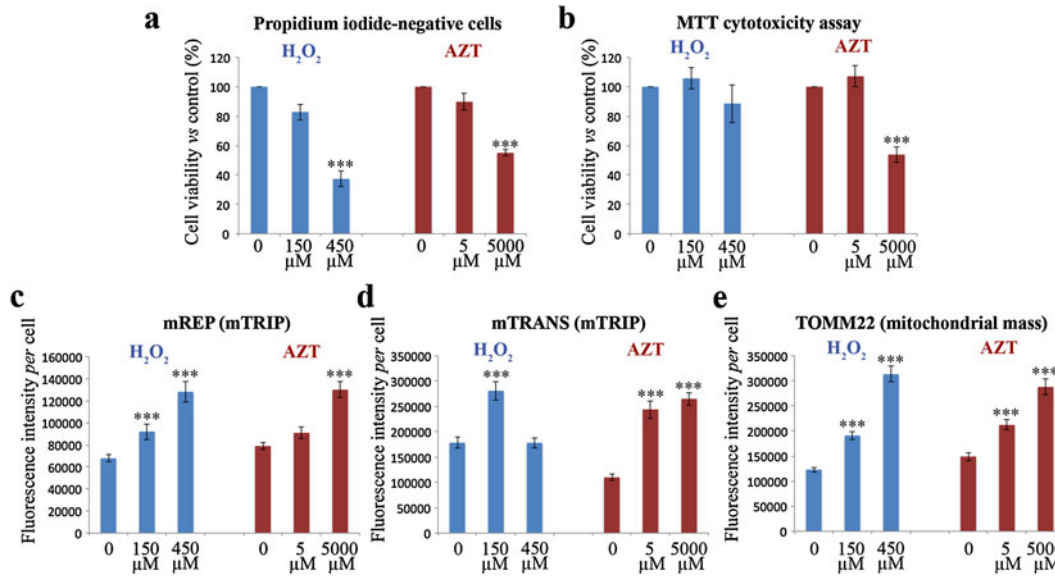

Fig. 4 mTRIP is a sensitive assay for detecting altered mtDNA processing at sublethal doses of H_2O_2 and AZT. As a proof of principle of mitochondrial impairment detected by mTRIP, HeLa cells were treated for 24 h with low and high doses of either the strong oxidizer H_2O_2, which damages cell components and also reduces the mtDNA content [17], or AZT (azidothymidine), an anti-HIV nucleoside analog that causes myopathy after long-term treatment [18]. In parallel, cell viability was assessed, using the propidium iodide (PI) assay and the MTT assay [18]. PI is an intercalating agent that is generally excluded from viable cells, and MTT is a tetrazolium dye that assesses the cellular metabolic activity, which is considered to some extent proportional to cell viability [18], through the levels of NAD(P)H-dependent cellular oxidoreductases. (**a**) Percent of PI-negative (viable) cells in the presence of increasing concentrations of either H_2O_2 or AZT, compared to untreated controls (0). (**b**) Metabolic activity, evaluated with the MTT assay, in cells treated with H_2O_2 or AZT, and expressed as percent of untreated controls (0). (**c**, **d**) Quantification of fluorescence intensity per cell of the mTRIP probe mREP (c) that labels initiation of mtDNA replication, and the mTRANS probe (d) that labels the global mtRNA content. (**e**) Quantification of fluorescence intensity per cell of the immunomarker TOMM22 (or TOM22), a mitochondrial outer membrane protein, used as indicator of the mitochondrial mass [14]. These results show that high doses (450 µM) of H_2O_2 result in a significant loss of cell viability, as expected, with PI assay (panel a) but not with the MTT (panel **b**) assay. Under these conditions, mTRIP displays a large increase of mREP (panel c) but not of mTRANS (panel **d**) signal compared to untreated controls. Similarly, high doses (5 mM) of AZT result in a significant loss of cell viability by PI and MTT assessments, and mTRIP displays a large increase of mREP and mTRANS signal. Taking into account the increase of the mitochondrial mass (immunofluorescence of the outer membrane protein TOM22, panel e), both treatments resulted in globally altered mtDNA processing activity, evaluated by mTRIP. Conversely, low dose (150 µM) of H_2O_2 did not result in loss of viability with either the PI or the MTT assays, whereas resulted in increased mREP and mTRANS signal compared to untreated controls. In a similar situation, low dose (5 µM) of AZT did not affect cell viability, detected by PI and MTT assays, whereas mTRIP resulted in increase of the mTRANS signal. Low doses of AZT did not affect the mREP signal. Taking into account that both treatments also augmented the mitochondrial mass, low doses of either compound resulted in altered mtRNA content and mtDNA initiation of replication. Thus mTRIP reveals alterations of mtDNA processing also at sublethal doses of these cytotoxic compounds. In c–e, quantification was performed on 30 cells per condition, from three independent experiments. Note that mTRIP detects mitochondrial alterations at high and low doses of H_2O_2 and AZT, whereas cytotoxicity, evaluated with standard methods, is detected only at high doses of these compounds. $N = 3$ independent experiments per conditions. $P < 0.001$ (***) *versus* untreated cells (0), according to the *T*-test

doses of the oxidant hydrogen peroxide (H_2O_2) and AZT (azidothymidine, a nucleoside analog used as antiretroviral), below the threshold of cytotoxicity detected with standard tests. As a control, at higher doses of these molecules, standard tests revealed cytotoxicity, and mTRIP detected extensive alterations of mitochondrial parameters. Thus, mTRIP is not conceived to reveal cell death but is highly sensitive to alteration of mitochondrial parameters at sublethal doses of drugs. With these characteristics, mTRIP is of potential interest for evaluating the impact of long-term treatments that affect cell function without immediate risk of cell death.

In conclusion, mTRIP is a powerful tool for fundamental studies of mtDNA processing linked to mitochondrial function, for assessing impaired mitochondrial function in disease, and also evaluating sublethal cytotoxicity.

2 Materials

All solutions must be prepared using ultrapure water. Disposing waste materials must be done according to waste disposal regulations.

2.1 Probe Preparation

1. Lysis buffer to extract total genomic DNA: 0.2 % SDS, 5 mM EDTA, 0.2 mg/mL Proteinase K in 1× phosphate buffered saline (PBS) buffer.
2. *LA Taq* DNA polymerase.
3. Sodium acetate 3 M, pH 5.2, cold ultrapure isopropanol and ultrapure water.
4. Primers for PCR amplification of mTRANS from total genomic DNA:

   mTRANS is a mix of three DNA probes: probe1, probe 6, and probe 11.

   Primers for PCR amplification of DNA probe 1 (961 nt):

   Forward 5′-ACCAGACGAGCTACCTAAGAACAG-3′

   Reverse 5′-CTGGTGAAGTCTTAGCATGT-3′

   Primers for PCR amplification of DNA probe 6 (1,118 nt):

   Forward 5′-CTACCACACATTCGAAGAACC-3′

   Reverse 5′-CGTTCATTTTGGTTCTCAGGG-3′

   Primers for PCR amplification of DNA probe 11 (1,420 nt):

   Forward 5′-CATACCTCTCACTTCAACCTC-3′

   Reverse 5′-TGAGCCGAAGTTTCATCATGC-3′

5. Primers for PCR amplification of mREP from total genomic DNA:

   Coordinates of the primers are according to NC_012920 GenBank.

   mREP (98 nucleotides, nt): coordinates 446–544.

   Forward 5′-ACATTATTTTCCCCTCCC-3′

   Reverse 5′-GGGGTATGGGGTTAGCAG-3′

6. PCR products clean-up system kit.
7. Labeling of purified mTRANS and mREP PCR products. It is strongly recommended to label the DNA probes by Nick-translation using Atto425 or Atto488 or Atto550 or Atto647 NT Labeling Kit (JenaBioscience) (*see* **Note 1**).

Storage of labeled and purified mTRANS and mREP probes: aliquoted at −20 °C in the dark.

2.2 Cell Treatment

1. Tissue culture plates 6-well (12-well or 24-well plates are alternatives for culture of rare primary cells or slow growing cells).
2. Glass cover slips (18 mm diameter or other size, according to diameter of the culture plate).
3. 2 % Paraformaldehyde (PFA) in PBS: mix 5 mL of 16 % PFA with 35 mL of PBS. Store at 4 °C for a maximum of 2 weeks.
4. Permeabilization buffer: 0.5 % Triton X-100 in PBS. Mix 50 μL of 100 % Triton X-100 with 9,550 μL PBS.
5. 20× saline sodium-citrate (SSC) buffer: 3 M NaCl, 300 mM Na_3Citrate.2 H_2O, pH 7.0. For 20× SSC preparation, dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of ultrapure water. Adjust the pH to 7.0 with 14 N solution of HCl. Adjust the volume to 1 L with ultrapure water. Sterilize by autoclaving. Store at room temperature for up to 6 months.
6. Pre-treatment buffer for permeabilized cells: 50 % formamide/2× SSC in PBS. Store at room temperature for up to 6 months, in the dark.
7. Denaturation buffer for permeabilized cells: 70 % formamide/2× SSC in PBS. Store at room temperature for up to 6 months, in the dark.

2.3 mTRIP Hybridization

1. Parafilm.
2. 100 ng/μL salmon sperm DNA. Dilute stock salmon sperm DNA with PBS. Store at −20 °C.
3. Hybridization buffer: 10 % dextran sulfate/50 % formamide/2× SSC in PBS. Store at room temperature for up to 6 months, in the dark (*see* **Note 2**).
4. Washing buffer A: 2× SSC in PBS.

5. Washing buffer B: 1× SSC in PBS.
6. Washing buffer C: 0.1× SSC in PBS.

2.4 mTRIP Coupled to Immunofluorescence

1. Parafilm.
2. Blocking buffer: 5 % bovine serum albumin (BSA) in PBS. Store at 4 °C, stable for several weeks (*see* **Note 3**).
3. Primary antibody (*see* **Note 4**). We recommend unconjugated rabbit polyclonal anti-TOMM22 to label mitochondria.
4. Fluorescent conjugated secondary antibody (*see* **Note 5**). We recommend Cy5 conjugated goat secondary anti-rabbit antibody (or another Cy Dye conjugate).
5. Hoechst 33342 or DAPI.

2.5 mTRIP Coupled to MitoTracker®

MitoTracker® Probes (Life technologies). Choose a MitoTracker probe according to spectral characteristics compatible with the successive mTRIP labeling. We recommend MitoTracker® Deep Red (655 nm).

2.6 Imaging Equipment

1. Confocal microscope.
2. Optional for 3D imaging: 3D-reconstruction imaging software.
3. Quantification—imaging software such as Image J, or equivalent.

3 Methods

mTRIP principles are summarized in Fig. 2 and representative mTRIP staining in a human cell is shown in Fig. 3.

3.1 Extraction of Total Genomic DNA

This includes nuclear and mitochondrial DNA. We recommend use of primary cells such as IMR90 at early passages (and not cancer-derived or immortalized cell lines) (*see* **Note 6**).

1. Add 200 µL of fresh lysis buffer to cell pellet.
2. Incubate for 3 h at 50 °C.
3. Add 20 µL of 3 M sodium acetate (pH 5.2).
4. Add 200 µL of cold ultrapure isopropanol.
5. Incubate for 15 min at −20 °C.
6. Centrifuge at 16,100 × *g* at 4 °C for 30 min.
7. Carefully discard the supernatant.
8. Add 500 µL of 70 % ethanol.
9. Centrifuge at 16,100 × *g* at 4 °C for 5 min.
10. Carefully discard the supernatant.
11. The pellet should be transparent if the extraction was correctly done. Dry the DNA pellet for 5 min at room temperature.

12. Resuspend the DNA pellet in at least 100 µL of ultrapure water.
13. Incubate at 4 °C overnight to ensure full resuspension.
14. Quantify the total genomic DNA. Dilute in ultrapure water, if needed.
15. Store at −20 °C.

3.2 Preparation of the Fluorescent DNA Probe

1. PCR amplify the mTRANS probe 1 using at least 100 ng/µL of total genomic DNA and *LA Taq* DNA polymerase. We recommend an annealing temperature of 56 °C and an extension temperature of 68 °C.
2. PCR amplify the mTRANS probe 6 using at least 100 ng/µL of total genomic DNA and *LA Taq* DNA polymerase. We recommend an annealing temperature of 56 °C and an extension temperature of 68 °C.
3. PCR amplify the mTRANS probe 11 using at least 100 ng/µL of total genomic DNA and *LA Taq* DNA polymerase. We recommend an annealing temperature of 56 °C and an extension temperature of 68 °C.
4. PCR amplify the mREP probe using at least 100 ng/µL of total genomic DNA and *LA Taq DNA* polymerase. We recommend an annealing temperature of 56 °C and an extension temperature of 68 °C.
5. Purify the PCR products through 2 % agarose gel electrophoresis and then conventional gel and PCR products clean-up kit.
6. Estimate the amount of the purified PCR product. **It is essential to obtain 1 µg of PCR product at a concentration of 75 ng/µL or higher.** If this amount is not reached, the DNA product can be concentrated by sodium acetate and isopropanol precipitation, then resuspended in the appropriate volume of ultrapure water to reach the required concentration. It is nevertheless recommended to repeat the PCR amplification to obtain the appropriate yield rather than concentrate more diluted preparations.

   Be careful, as the following steps are dedicated to DNA probe fluorescent labeling by Nick-translation and are essential for mTRIP. Consider that for mTRIP probes no other technique of DNA labeling than Nick-Translation has been tested to date.
7. Select the fluorescence label for mREP and mTRANS DNA probes (*see* **Note 7**). If mTRIP is coupled to immunofluorescence, or MitoTracker, resulting in four color channels (which include the staining with Hoechst 33342), the recommended choice is: mREP label with Atto550 NT kit, mTRANS label with Atto488 NT kit, immunofluorescence

with a Cy5-conjugated secondary antibody (or, alternatively, mitochondria labeling by MitoTracker® Deep Red FM), and Hoechst.

8. Prepare the mTRANS DNA probe by mixing 333 ng of mTRANS probe 1, 333 ng of mTRANS probe 6, and 333 ng of mTRANS probe 11 (same amount of the three probes) for a final amount of 1 μg DNA, in maximum volume of 14 μL, and in a 0.2 mL thin-wall 8-tube strip with caps for a regular thermal cycler (PCR machine). If the volume is smaller, adjust to 14 μL with PCR-grade water from the kit.
9. Prepare the mREP DNA probe using 1 μg of pure PCR product in a maximum volume of 14 μL, and in a 0.2 mL thin-wall 8-tube strip with caps for a regular thermal cycler (PCR machine). If the volume is smaller, adjust to 14 μL with PCR-grade water from the kit.

The following steps are identical for mREP and mTRANS fluorescence labeling:

10. Gently mix 14 μL of template DNA with 2 μL of 10× NT labeling buffer, on ice.
11. Add 2 μL of Atto488 or Atto550 NT labeling mix.
12. Add 2 μL of 10× Enzyme mix.
13. Gently mix to ensure sample homogeneity.
14. Incubate the mix for 90 min at 15 °C, for example in a regular thermal cycler, in the dark.
15. Add 5 μL of NT Kit Stop buffer to stop the reaction.
16. Transfer the reaction mix to a 1.5 mL tube.
17. Add 2 μL of 3 M sodium acetate at pH 5.2.
18. Add 14 μL of cold ultrapure isopropanol.
19. Gently mix the sample.
20. Incubate at −20 °C for 15 min in the dark.
21. Centrifuge at 16,100 × *g* for 30 min at 4 °C.
22. Discard the supernatant.
23. Add 500 μL of 70 % ethanol.
24. Centrifuge at 16,100 × *g* for 5 min at 4 °C.
25. Discard the supernatant.
26. Add 500 μL of 70 % ethanol.
27. Centrifuge at 16,100 × *g* for 5 min at 4 °C.
28. Discard the supernatant.
29. Resuspend the fluorescence-labeled DNA probe in 50 μL of ultrapure water for a final concentration of 20 ng/μL.
30. Store the labeled DNA probe at −20 °C in the dark.

3.3 Pre-hybridization of the DNA Probe

To reduce bench timing, Subheading 3.3 *(Pre-hybridization of the DNA probe)* can be performed in parallel with Subheading 3.4 *(Preparation of cells on cover slip)*.

1. Selection 1. **ONE probe**, preparation of the reaction mix in a 1.5 mL tube:
   If only one probe (e.g., either mREP or mTRANS) is hybridized, the reaction mix is done in a final volume of 25 μL per cover slip. Gently mix 2 μL of 20 ng/μL fluorescent DNA probe (40 ng DNA probe per cover slip at a final concentration of 1.6 ng/μL) with 4 μL of 100 ng/μL salmon sperm DNA (400 ng of salmon sperm DNA are added for 40 ng of DNA probe, and per cover slip) and 19 μL of hybridization buffer.
2. Selection 2. **TWO probes**, preparation of the reaction mix in a 1.5 mL tube:
   If two probes (mREP and mTRANS) are hybridized, the reaction mix is done in a final volume 25 μL per *c*over slip. Gently mix 2 μL of 20 ng/μL fluorescent mREP probe (40 ng of DNA probe are used per cover slip; final concentration 1.6 ng/μL) and 2 μL of 20 ng/μL fluorescent mTRANS probe (40 ng DNA probe per cover slip; final concentration 1.6 ng/μL) with 8 μL of 100 ng/μL salmon sperm DNA (800 ng salmon sperm DNA are added for 2 × 40 ng of DNA probes and per cove slip) and 13 μL of hybridization buffer.
3. Denaturation: incubate the reaction mixture at 80 °C for 10 min in the dark.
4. Pre-cooling: transfer the tube containing the denatured reaction mix at 37 °C for at least 30 min in the dark. This time can be extended to 1 h maximum (again at 37 °C in the dark). This step helps lowering unspecific binding of salmon sperm DNA *versus* specific binding of DNA probes. Keep the denatured reaction mix under these conditions until the end of Subheading 3.4 (Preparation of cells on cover slip).

3.4 Preparation of Cells on Cover slip

1. Cell culture: culture cells under appropriate conditions on plates that contain a clean slide at the bottom of each well. Ideally, cells should reach no more than 50–60 % confluence to avoid plans with packed cells during microscope analysis.
   If mTRIP is coupled to MitoTracker staining, incubate live cells with MitoTracker® (for instance Mitotracker ® Deep Red) for 1 h under appropriate culture conditions (*see* **Note 8**).
2. Discard the culture medium.
3. Wash cells once with PBS.
4. Add 2 % PFA kept at room temperature.
5. Incubate for 20 min at room temperature (RT).
6. Discard the PFA.

7. Wash two times with PBS. At this step, PFA-fixed cells can be stored in PBS for several weeks at 4 °C.
8. Discard the PBS from (fixed) cells on the cover slip.
9. Permeabilize cells with 0.5 % Triton X-100 in PBS for 5 min at 4 °C.
10. Discard the Triton X-100.
11. Wash four times with PBS.
    OPTIONAL: if required, at this step permeabilized cells can be incubated with specific nucleases for the time recommended by the manufacturer (general recommendation: 1 h at 37 °C). After incubation, wash four times with PBS (*see* Note 9).
12. Discard the PBS.
13. Pre-treat permeabilized cells by adding pre-treatment buffer (50 % formamide/2× SSC in PBS).
14. Incubate for 30 min at room temperature.
15. Discard the pre-treatment buffer.
16. Add 70 % formamide/2× SSC in PBS.
17. Put the culture plate (with, at the bottom of each well, a slide with pre-treated permeabilized cells) on the top of a metal block heater for 5 min at 75 °C, to denature the sample.
18. Immediately transfer the culture plate on ice (0–4 °C) and keep on ice for at least 1 min but not longer than 10 min (*see* **Note 10**).

3.5 mTRIP Hybridization

1. Put a 25 μL drop of denatured and pre-cooled reaction mix which contains DNA probe(s) on an appropriate surface of parafilm (at least 3.6 × 3.6 cm for 18 mm diameter slide). For multiple labeling, use a single surface of parafilm with enough room for multiple and well separated drops.
2. Invert the coverslip upside down, with the side covered by cells (previously denatured sample) directly facing the drop.
3. Incubate at 37 °C for 15 h either on top of a heating metal block in the dark (cover with a non-transparent plastic top to keep dark) or on a humid chamber at 37 °C (always in the dark).

3.6 mTRIP Washing

1. After 15 h of incubation, remove the cover slip from the parafilm surface and put it back into a clean cell culture plate (e.g., a 6-well plate).

   The following steps must be performed in the dark:
2. Wash twice with 2× SSC in PBS for 2 min at RT with gentle shaking.

3. Wash twice with 1× SSC in PBS for 2 min at RT with gentle shaking.
4. Wash twice with 0.1× SSC in PBS for 2 min at RT with gentle shaking.
5. Wash twice with PBS for 2 min at RT with gentle shaking.

At this point, either label the sample by immunofluorescence (step 3.7, mTRIP coupled to immunofluorescence) or do not label the sample by immunofluorescence (this is also the case for MitoTracker labeling) and perform the following:

6. Incubate the cover slip for 1 h in the dark with 10 μg/mL of Hoechst 33342 in PBS.
7. Wash five times with PBS.
8. Mount the cover slip on a clean and dry glass slide using mounting medium or a drop of 50 % glycerol in PBS.
9. Seal the slide with nail polish and keep the mounted slide in a dark and clean box either at RT if confocal analysis is to be done on the same day, or at 4 °C until confocal analysis is done (it is recommended to analyze the slide no later than 2 weeks after labeling).

3.7 Coupled to Immunofluore scence

Follow the immunofluorescence (IF) protocol suggested below (*see* **Note 11**).

1. Incubate the cover slip with 5 % BSA in PBS at RT for 1 h in the dark.
2. Wash twice in PBS for 2 min at RT with gentle shaking.
3. Dilute the primary antibody, anti-TOMM22, 1:200 in 1 % BSA and PBS. We recommend this antibody for assessing the mitochondrial network.
4. Put a 50 μL drop of diluted primary antibody mix on an appropriate surface of parafilm.
5. Put the cover slip upside down, with the side covered by cells directly facing the drop.
6. Incubate at RT for 1 h in the dark. The incubation time depends on the primary antibody used (follow the manufacturer's recommendations).
7. Wash three times in PBS for 2 min at RT with gentle shaking.
8. Dilute secondary antibody (generally 1:1000, follow the manufacturer's recommendations) in 1 % BSA and PBS, and add 10 μg/mL of Hoechst 33342.
9. Put a 50 μL drop of diluted secondary antibody mix on an appropriate surface of parafilm.
10. Put the cover slip upside down, with the side covered by cells directly facing the drop.

11. Incubate at RT for 1 h in the dark.
12. Wash five times in PBS for 2 min at RT with gentle shaking.
13. Mount the cover slip on a clean and dry slide using mounting medium or a drop of 50 % glycerol in PBS.
14. Seal the slide with nail polish and keep the mounted slide in a dark and clean box either at RT if confocal analysis is to be done the same day, or at 4 °C until confocal analysis is done (it is recommended to analyze the slide no later than 2 weeks after labeling) (*see* **Note 12**).

3.8 Imaging Acquisition and Fluorescence Intensity Quantification

1. Acquisition is done with confocal or classical fluorescence microscope (*see* **Note 13**).
2. One can do either 2-dimension (2D) or 3-dimension (3D) acquisition. In the last case, 3D-reconstruction of the image should follow 3D acquisition.
3. If the acquired images are in tiff format (.tiff), the fluorescence intensity per cell can be quantified using Image J software (or equivalent). A 3D-reconstructed human cell stained by mTRIP is shown in Fig. 3 (panels a, b, c).
4. A comparison of mTRIP with propidium iodide (PI) and MTT assays [16] for the detection of sublethal cytotoxicity following H_2O_2 [17] or AZT (azidothymidine) [18] treatment is shown in Fig. 4.

4 Notes

1. Labeling of DNA probes has been tested only with Nick Translation. It is possible that other DNA probes labeling techniques are compatible with mTRIP.
2. The hybridization buffer should be prepared several days (at least 2 days) before the experiment since dextran sulfate takes long time to dissolve.
3. Alternative blocking buffers can also be used.
4. Unconjugated rabbit polyclonal anti-TOMM22 is used to detect the mitochondrial network. However, the mitochondrial network can be detected with other primary antibodies, and immunolabeling can be used to mark other cellular structures or proteins of interest.
5. The choice of the secondary antibody-conjugated fluorescence depends on the available spectra after exclusion of the fluorophore(s) used for DNA probes (mTRIP). All combinations compatible with available fluorophores and the equipment (fluorescence filters) of the microscope can be used.

6. Cancer and immortalized cell lines may have mutated or highly heteroplasmic (coexistence of more than one mitochondrial genome) mitochondrial genomes.
7. The fluorescence wavelengths of the DNA probes can be different from the ones used here. Appropriate fluorophores must be selected *ad hoc*.
8. In this case, use mREP-Atto550 and mTRANS-Atto-488 for mTRIP labeling, and Hoechst.
9. Treatment with nucleases is conceived to identify the DNA or RNA components of the mTRIP signal. DNaseI (recommended 100 U/mL) or RNAseA (recommended 100 μg/mL) treatment identifies the DNA and RNA components of the mTRIP labeling, respectively. Treatment with RNAseH (recommended 100 U/mL) identifies RNA/DNA structures, generally at replication origins and in transcription bubbles. For the combined use of nucleases and the respective readouts on mitochondrial nucleic acids by mTRIP, *see* Ref. 14. Probes mREP and mTRANS have been shown to label only DNA and RNA, respectively [14]. The use of nuclease is recommended for new probes conceived to target a specific region of the mitochondrial genome or a specific transcript.
10. Do not keep denatured samples at RT as this condition will interfere with the hybridization step. Keep the samples always on ice until hybridization.
11. The favorite IF procedure, if different from the one described here, can alternatively be performed.
12. mTRIP can be used with paraformaldehyde-fixed human cells and paraffin-embedded human tissue sections (method not described here).
13. Reliable quantification requires fluorescence measurement within the cell volume. Cell surface (2D-acquisition) can also be acquired instead, but the corresponding quantification will not measure the total cell fluorescence. In case of 2D-acquisitions it is recommended to analyze a high number of individual cells (>50 cells per condition, from three independent experiments), in particular if labeling is not uniform and/or cells have irregular shapes.

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