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Single molecule mtDNA fiber FISH for analyzing numtogenesis

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Abstract

Somatic human cells contain thousands of copies of mitochondrial DNA (mtDNA). In eukaryotes, natural transfer of mtDNA into the nucleus generates **nuclear mitochondrial DNA (NUMT)** copies. We name this phenomenon as “*numtogenesis*”. Numtogenesis is a well-established evolutionary process reported in various sequenced eukaryotic genomes. We have established a molecular tool to rapidly detect and analyze NUMT insertions in whole genomes. To date, NUMT analyses depend on deep genome sequencing combined with comprehensive computational analyses of the whole genome. This is time consuming, cumbersome and cost prohibitive. Further, most laboratories cannot accomplish such analyses due to limited skills. We report the development of single-molecule mtFIBER FISH (fluorescence in situ hybridization) to study numtogenesis. The development of mtFIBER FISH should aid in establishing a role for numtogenesis in cancers and other human diseases. This novel technique should help distinguish and monitor cancer stages and progression, aid in elucidation of basic mechanisms underlying tumorigenesis and facilitate analyses of processes related to early detection of cancer, screening and/or cancer risk assessment.

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Introduction

Mitochondrial oxidative phosphorylation (OXPHOS) is controlled by two genomes: mitochondrial DNA (mtDNA) and the nuclear DNA (nDNA). In addition to ATP production through OXPHOS, mitochondria play a central role in cell proliferation, cell signaling, metabolite homeostasis and apoptosis and are involved in various human diseases [1–3]. Human mtDNA is very small (16,569 bp) and contains one single control region called the D-loop that controls mtDNA replication and transcription of mtDNA-encoded OXPHOS genes. The entire protein coding capacity of the mitochondrial genome is dedicated to the production of 13 protein subunits. These 13 proteins constitute the various subunits that make up four OXPHOS complexes (Complex I, III, IV, and V). Complexes I to IV are electron transport proteins, and Complex V is an ATP synthase [2,4]. Nuclear DNA encodes the remaining OXPHOS complexes (73 subunits). Subunits encoded by nuclear DNA (38 for Complex I, 10 each for Complex III and IV and 15 for Complex V) are translated in the cytosol and imported into the mitochondrial compartment [2,5–7]. All four subunits constituting complex II are entirely encoded by nDNA. Human somatic cells contain thousands of copies of mitochondrial DNA (mtDNA) [8]. In contrast, a human oocyte contains up to five million mitochondria and a correspondingly large number of mtDNA copies [9]. The mtDNA copy number of individual cells not only varies with the type of cell and tissue but also with age. The number is altered during cell death, cell differentiation, hormonal changes and exercise [10]. Our studies demonstrate that mtDNA plays an important role in carcinogenesis [1,7,11–14]. Consistent with our studies other laboratories report a decrease in mtDNA content in breast [15,16], renal [17], hepatocellular [10,18], gastric [19] and prostate tumors [20]. In tumors depletion of mtDNA is accompanied by a decrease in OXPHOS levels [21]. In breast cancer patients, depletion of mtDNA correlates with tumor progression and a poor prognosis [22]. A study also suggests that depletion of mtDNA is a risk factor for renal cell carcinoma [23]. We have provided evidences that in African-Americans low mtDNA content is a risk of aggressive cancer [24]. These studies suggest that mito-nuclear interactions are involved in the development of cancer. However the role of mtDNA integration into the nuclear genome remains unexplored. Transfer of mtDNA into the nucleus generates nuclear mitochondrial DNA (NUMT) copies. NUMT sequences are reported in at least 85 sequenced eukaryotic genomes [25]. These include human, plant, yeast, fruit fly, *Plasmodium*, *Caenorhabditis* and other species [25]. Although NUMTs are transcriptionally inactive, recent findings have suggested that in mammals integration of NUMTs is non-random [26,27]. The human genome contains NUMT, with mtDNA identities ranging from 64 to 100% [28,29]. We describe this phenomenon of insertion of mtDNA fragments into nuclear genome as ‘*numtogenesis*’. To date neither the relevance of numtogenesis in human pathology nor the mechanisms underlying numtogenesis is clear.

FIBER-FISH is a technique that permits high resolution mapping of genes and chromosomal regions on single fiber(s) of DNA, allowing physical location of DNA probes down to a resolution of 1000 bp. This technique also allows assessment of gaps, overlaps, segmental duplications and copy number variants in the nuclear genome. The method consists of releasing DNA fibers from cell nuclei and stretching and fixing them on a microscope slide

prior to hybridization [30–32]. An improvement in producing uniformity in DNA stretching and reproducibility can be achieved by a molecular combing which uses the action of a receding air/water meniscus to extend and align DNA molecules attached at one end to a glass surface [32,33]. Here we have developed FIBER-FISH to detect mtDNA fragments (NUMT) in the nuclear genome. We utilized state of the art FIBER-FISH technology and optimized it for mtDNA detection. We have named this as mtFIBER-FISH which will allow rapid analyses of NUMT in cancer cell lines and primary tumors and advance basic and translational cancer research.

Materials and methods

Mitochondria and nuclei isolation

Pure mitochondrial fractions were prepared from MCF-12A and MDA-MB-231 cells according to the previously described protocol with some modifications [34]. Briefly, cells growing in 150 mm tissue culture plates were scraped into 5 ml of PBS and centrifuged at $800\times g$ for 5 min. All further manipulations were performed at 4°C. The cell pellet was dissolved in 5 ml of solution A (20 mM Hepes-KOH pH 7.6, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM PMSF, and 2 mg/ml bovine serum albumin), and homogenized with a glass homogenizer for 40–50 strokes. The homogenized solution was then again centrifuged at $800\times g$ for 5 min and the supernatant was collected and further centrifuged at $10,000\times g$ for 10 min. The supernatant was removed and the pellet was re-suspended in solution B (solution A without bovine serum albumin). After a low speed clarifying spin ($800\times g$, 5 min), the supernatant was further centrifuged at $10,000\times g$ for 10–20 min to collect purified mitochondria. The mitochondrial pellet was dissolved in sucrose buffer (10 mM Hepes pH 7.6 and 0.5 M sucrose) and used to make single strand DNA fibers of the mitochondrial genome.

To avoid mtDNA contamination in nuclei preparation, we purified nuclear fractions. Briefly, wild-type MCF-12A/MDA-MB-231 cells were lysed using lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA) containing 10% IGEPAL detergent for 10 min at room temperature and by centrifugation at $15000\times g$ for 3 min intact nuclei were pelleted out. To completely avoid the mitochondrial contamination in nuclear pellet, the lysis step was repeated several times. The purity of the nuclear fraction was ascertained by performing western blot for mitochondrial encoded Cytochrome oxidase II (COXII) protein.

Probe preparation

The primer pairs used to amplify the specific region of mtDNA are listed in Table 1. Amplified PCR products were purified using Centri-Sep spin columns (Princeton Separations, NJ, USA). The probe labeling of each purified PCR product was accomplished by the nick translation according to the manufacturer's protocol (Roche, Indianapolis, IN). The labeling mixture was composed of 5 μ l of 0.2 mM dATP, dGTP and dCTP each, 1 μ l of biotin-16-dUTP, dioxigenin-11-dUTP or dinitrophenol (DNP)-11-dUTP, instead of dTTP, 1 μ g of probe DNA, and 5 μ l of DNaseI/DNA polymerase. The final volume was adjusted to 50 μ l with distilled water in a 1.5 ml microfuge tube that was placed on ice, then mixed. The mixture was centrifuged for 5 s incubated at 15 °C for 120 min, and at 65 °C for 10 min, and

placed at room temperature for 15 min. Then, 6.25 μ l of 4 M ammonium acetate and 5 μ l of 100% ethanol were added to stop the reaction. The mixture was centrifuged for 5 min at 12,000 rpm and the supernatant was discarded. The pellet was diluted with 20 μ l of 100% formamide.

Slide preparation

Lambda or mtDNA fibers were prepared on poly-L-lysine slides (Sigma, St. Louis, MO). Five microliters of resuspended DNAs was placed in the center of a slide, and 15 μ l of lysis buffer (2% sarkosyl, 0.25% SDS, 50 mM Tris, pH 7.4, 50 mM EDTA, pH 8.0, and 0.5% Triton X-100) was added. Slides were incubated for 10 min at 37 °C, at which time a 22 $\times$ 22-mm cover slip was gently placed over the DNA suspension. DNA fibers were fixed at 60 °C for 30 min, and cover slips were fixed in a 3:1 solution of 100% ethanol:glacial acetic acid and dried at 60 °C for 15 min.

Extension of nDNA fibers was carried out by following the method of Jackson et al. [35]. Two micro-liters of the nuclei suspension were deposited on one end of a Poly-L-lysine slide (Sigma) and dried briefly. After this, 10 μ l of lysis buffer [0.5% (w/v) SDS, 5 mM EDTA, plus 100 mM Tris (pH 7.0)] was placed on the nuclei preparation and the preparation was incubated at room temperature for 4 min; the DNA fibers were then extended by use of a cover slip. The slides were completely air-dried, fixed in 100% ethanol-glacial acetic acid (3:1) for 2 min, and heated at 60 °C for 30 min.

Hybridization and detection

Fiber-FISH hybridization and detection were carried out as described previously [35,36]. Briefly, Fiber-FISH hybridization was performed at 37 °C overnight with a probe mixture containing 50 ng DNA probe, 50% formamide, 10% dextran sulphate, 10 mM EDTA, 3 M NaCl and 5 μ g of salmon sperm DNA (Sigma). For the post hybridization wash, the slides were washed twice in 2X SSC for 5 min each at room temperature. Then, the slides were washed in 50% formamide in 2X SSC and 2X SSC at 42 °C for 10 min each, respectively. Digoxigenin-labeled probes were detected using mouse anti-digoxigenin antibody (1:200; Roche) followed by rabbit anti-mouse antibody conjugated to Alexa 568 (1:200; Invitrogen, Carlsbad, CA). Biotin labeled probes were detected with goat anti-biotin conjugated with avidin (1:200; Vector Laboratories, Burlingame, CA) followed by streptavidin-Alexa 488 (1:200; Invitrogen, Carlsbad, CA). The DNP-labeled probe was detected with rabbit anti-DNP, followed by amplification with a chicken anti-rabbit Alexafluor 647 antibody.

Image analysis

The Fiber-FISH images were captured with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy LLC, Thornwood, NY) using a cooled CCD camera CoolSNAP HQ2 (Photometrics, Tucson, AZ) and AxioVision 4.8 software. The final contrast of the images was processed using Adobe Photoshop CS5 software. Fiber-FISH signal measurements were performed using Image J software.

Results and discussion

Determination of fine mapping resolution

Fiber-FISH is a specialized technique for high resolution (few kilobases) analysis of DNA fibers, but its practical application is often limited due to its low signal-to-noise ratio, long exposure times and labor intensive sample preparation. Large genomic DNA clones such as bacterial artificial chromosome (BAC) and yeast artificial chromosomes (YAC), can be spread on glass slides and used for fluorescence in situ hybridization mapping [37–40]. On slides, DNA prepared from such genomic DNA clones extends approximately 3.0 kb/ μ m. Thus, the Fiber-FISH provides fine-mapping resolution of up to a few kilobases [41].

To test the mapping resolution, we used lambda DNA molecules, which have widely been employed for sizing of DNA fragments in agarose gels. DNA fingerprint analysis using *Hind*III digestion yielded six DNA fragments, 23 kb, 11 kb, 9 kb, 2.3 kb, 2.0 kb and 0.5 kb in order length (Fig. 1A). Lambda DNA fragments were grouped into three pools based on their location on the *Hind*III restriction map (Fig. 1B). These pools were labeled, and their distributions on individual lambda molecules were determined by Fiber-FISH mapping as described by Jackson et al. (1999) [37]. Fig. 1C shows the lambda molecules that are labeled with three different colors: red, green and blue, which varied in length from 13.5 to 18 μ m (average 15.5 μ m; 46.5 kb). These results indicate that Fiber-FISH can be applied for positional and directional information of DNA clones at small genomic scales.

Development of mtDNA fiber FISH

In the field of numtogenesis, an important challenge is the lack of a rapid molecular technology to detect and analyze NUMT insertions in the whole genome. We have developed such a technique to detect and analyze NUMT insertions.

We analyzed NUMTs in MCF-12A/MDA-MB-231 cell lines. Probe containing a mixture of 13 PCR products covering the entire human mtDNA (Fig. 2A) was used for detecting individual mtDNAs and their nuclear copy of mtDNAs (NUMTs) on extended DNA fibers. To test the mtDNA probes, we first performed Fiber-FISH analysis with mtDNA fibers prepared from the mtDNA of a MCF-12A cell line (Fig. 2B). Visualization of mtDNA isolated from the MCF-12A cells showed that most mtDNA fibers showed a bead-and-string pattern with a super-coiled structure, and a few open circular mtDNAs were also detected (Fig. 2B). The Fiber-FISH technique was also used to detect the NUMTs of MCF-12A/MDA-MB-231 cells. The result showed FISH labeling patterns with different sized red-green signals, indicating the presence of variable NUMTs in MCF-12A (Fig. 2C)/MDA-MB-231 (Fig. 2D) cell lines. We do not yet know how NUMTs are generated. However, it is likely that degradation of mitochondria involving mitophagy, direct contact or fusion between mitochondrial and nuclear membranes and translocation of mitochondria in the nucleus may contribute to generation of NUMT.

Conclusions

Numtogenesis is an evolutionary conserved process reported in a large number of eukaryotic genomes [25]. We have developed a molecular technology to rapidly detect and analyze

NUMT insertions in the whole genome. The development of mtFIBER-FISH will aid in genetic validation of increased numtogenesis to investigate the likelihood of NUMT contribution to the development of cancer in humans, and identification of the size, sequence and specific insertion sites in human cancer genomes [42]. At a broader level, development of mtFIBER-FISH provides a molecular technology to rapidly detect and analyze NUMT insertions that open a new field, numtogenesis, and allow comprehension of the function of this process in cancer and other human diseases. Development of mtFIBER-FISH will provide researchers from other disciplines an advanced method for analyses of numtogenesis in other organisms and field.

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Abbreviations

mtDNA	Mitochondrial DNA
NUMT	Nuclear Mitochondria or mtDNA
FISH	Fluorescence in situ hybridization
OXPHOS	Oxidative phosphorylation

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Fig. 1. Visualization of lambda molecules. **A**, schematic representation of electrophoresis of lambda DNA digested using *Hind*III restriction enzyme. **B**, physical distribution of *Hind*III sites on lambda DNA. Red, green and blue bars represent the FISH probe mixture that used to detect each DNA fragment on intact lambda DNAs. **C**, Fiber-FISH patterns of lambda molecules. Bar, 10 μm.

Fig. 2. Fiber-FISH detection of mtDNA and NUMTs in MCF-12A/MDA-MB-231 cell lines. **A**, schematic representation of human mtDNA and FISH probe designations. **B**, Fiber-FISH labeling patterns of mtDNA prepared from a MCF-12A cell line. Insert in **B** represents the open circular structure of mtDNA of a MCF-12A cell. a-e represent the FISH probe labeling pattern that corresponds to the probe designation in **A**. **C–D**, Fiber-FISH patterns of NUMTs on nuclear DNA fibers prepared from MCF-12A (**C**) and MDA-MB-231 (**D**) cell lines. Arrows point to distinct NUMTs on nuclear DNA fibers. The PCR products known to hybridize to the human mtDNA were used as labels: M23, M1, M2, M9, M12, M13 (red); M3, M4, M5, M17, M19, M20, M22 (green). Bars, 5 μ m.

Table 1

Primers used for mitochondrial probe preparation. Primer sequence and each PCR product size used for probe preparation is described.

Probe	Primer sequences	PCR amplicon size (bp)	Tm
M1	CTCCTCAAAGCAATACACTG TGCTAAATCCACCTTCGACC	839	55
M2	CGATCAACCTCACCACCTCT TGGACAACCAGCTATCACCA	801	55
M3	GGACTAACCCCTATACCTTCTGC GGCAGGTCAATTTCACTGGT	859	55
M4	AAATCTTACCCCGCCTGTTT AGGAATGCCATTGCGATTAG	886	55
M5	TACTTCACAAAGCGCCTTCC ATGAAGAATAGGGCGAAGGG	831	55
M9	GAGGCCTAACCCCTGTCTTT ATTCCGAAGCCTGGTAGGAT	827	55
M12	ACGAGTACACCGACTACGGC TGGGTGGTTGGTGTAATGA	909	55
M13	TTTCCCCTCTATTGATCCC GTGGCCTTGGTATGTGCTTT	815	55
M17	TCACTCTCACTGCCCAAGGA GGAGAATGGGGGATAGGTGT	801	55
M19	AAACAACCCAGCTGTCCCTA TCGATGATGTGGTCTTTGGA	976	55
M20	ACATCTGTACCCACGCCTTC AGAGGGGTCAGGGTTGATTTC	969	55
M22	TGAAACTTCGGCTCACTCCT AGCTTTGGGTGCTAATGGTG	1161	55
M23	TCATTGGACAAGTAGCATCC GAGTGGTTAATAGGGTGATAG	809	55