bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

RESEARCH

Mito-seek enables deep analysis of mitochondrial DNA, revealing ubiquitous, stable heteroplasmy maintained by intercellular exchange

Anitha D Jayaprakash[1], Erica Benson[1], Raymond Liang[1], Jaehee Shim[1], Luca Lambertini[1], Mike Wigler[2], Stuart A Aaronson[1] and Ravi Sachidanandam[1*]

*Correspondence: ravi.mssm@gmail.com
[1]Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai , One Gustave L. Levy place, New York, 10029, USA
Full list of author information is available at the end of the article

Abstract

Eukaryotic cells carry two genomes, nuclear (nDNA) and mitochondrial (mtDNA), which are ostensibly decoupled in their replication, segregation and inheritance. It is increasingly appreciated that heteroplasmy, the occurrence of multiple mtDNA haplotypes in a cell, plays an important biological role, but its features are not well understood. Until now, accurately determining the diversity of mtDNA has been difficult due to the relatively small amount of mtDNA in each cell ($< 1\%$ of the total DNA), the intercellular variability of mtDNA content and copies of mtDNA pseudogenes in nDNA. To understand the nature of heteroplasmy, we developed Mito-seek, a novel technique that purifies and sequences mtDNA. Mito-seek yields high purity ($> 98\%$) mtDNA and its ability to detect rare variants is limited only by sequencing depth, providing unprecedented sensitivity and specificity. Using Mito-seek, we confirmed the ubiquity of heteroplasmy by analyzing mtDNA from a diverse set of cell lines and human samples. By applying Mito-seek to colonies derived from single cells, we showed that heteroplasmy is stably maintained in individual daughter cells over multiple cell divisions. Our simulations indicate that the stability of heteroplasmy can be facilitated by the exchange of mtDNA between cells. We also explicitly demonstrate this exchange by co-culturing cell lines with distinct mtDNA haplotypes. Our results shed new light on the maintenance of heteroplasmy and provide a novel platform to investigate various features of heteroplasmy in normal and diseased tissues.

Keywords: mitochondria; mtDNA; heteroplasmy; genetics

Background

Mitochondria are organelles present in almost every eukaryotic cell [1]. They enable aerobic respiration[2] to efficiently generate ATP, and play an important role in oxygen sensing, inflammation, autophagy, and apoptosis[3, 4].

Mitochondrial activity relies on over a thousand proteins, mostly coded on the nuclear DNA in humans[5], but genes from the mitochondrial genome, a small circular DNA (**mtDNA**), play a critical role in their function. In humans, the mtDNA is $\approx$ 17 kbp and codes thirteen proteins critical for the electron transport chain, along with twenty-two tRNAs, two rRNAs and a control region, called the displacement loop (D-loop) (Fig. 1)[6]. Their genetic code differs from the nuclear one hinting at a bacterial origin; in mammalian mitochondria, ATA codes for Methionine instead of Isoleucine, TGA codes for Tryptophan instead of the stop codon, and AGA, AGG code for stop codons instead of Arginine[7].

bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Each mitochondrion carries multiple mtDNA($5-10$)[8] and each cell contains hundreds to thousands of mitochondria, depending on the tissue[9]. Mitochondria are inherited solely from the mother and reproduce without recombination. Mutations in mtDNA have been linked to several genetic disorders including diabetes mellitus and deafness (DAD) and Leber's hereditary optic neuropathy (LHON)[10]. De novo mutations in mtDNA may also affect cell function.

Widespread heteroplasmy in normal human cells has been documented[11]. Diversity in heteroplasmy has also been inferred from whole-exome data of the 1000 genomes project[12], but, as we demonstrate below, careful work is needed to accurately measure heteroplasmy. Conflicting results from various studies have muddied the waters. For example, studies in mice have proposed that homoplasmy is the preferred state, and heteroplasmy is potentially deleterious[13, 14], another study showed that eventually tumors are expected to become homoplasmic, due to drift[15]. Variations in heteroplasmy can have important clinical implications[16], but its role in disease progression is uncertain[17]. Skewed transmission of heteroplasmic mtDNA, with only a few haplotypes of the mother appearing in the child, has also been implicated as a source of disease[18]. Thus, the role of heteroplasmy and its prevalence is not conclusively delineated, largely due to technical challenges.

Purifying mtDNA is essential for accurate determination of heteroplasmy, due to the nuclear-mtDNA pseudogene sequences (**Numts**) present in mammalian genomes[19] (Fig. 2). As with any other part of the nuclear genome, Numts exhibit variability and occur in variable copy numbers. Without purification, Numts contaminate the measurements of mtDNA variants, and introduce inaccuracies in the measures of heteroplasmy. Thus, the methods mentioned below are unable to accurately identify low-frequency variants in mtDNA.

Isolating mtDNA has long been a challenge. Primers specific to mtDNA have been used to either perform long-range PCR[20], or amplify hyper variable regions(HVR) in the D-loop[21]. In forensics and genealogy, allele-specific primer extensions (SNaPshot) are used for genotyping mtDNA[22]. Isolation of organelles by ultra-high-speed centrifugation has also been used, the yields are low along with contamination from fragmented nuclear DNA[23].

Recently, deep sequencing approaches have been used to study the mitochondrial genome. Long-range PCR has been used to capture mtDNA from complex DNA mixtures which is either sequenced after multiplexing[20] or by pyrosequencing[24]. Other approaches have included whole-genome sequencing of tumors[25], as well as whole-exome sequencing which includes off-target capture of mtDNA fragments[26, 12]. Whole genome sequencing can also estimate the number of mitochondrial genomes per nuclear genome[27]. Another study used a multitude of mtDNA-specific PCR primers to amplify short fragments (≈ 650 nt) for sequencing [11], and confirmed their results by re-sequencing the samples using two different techniques, a capture-based method and a pcr-based method that amplified longer fragments of mtDNA (a few kb). A new approach uses methyl-specific endonucleases MspJI and AbaSI to deplete nDNA that is likely to be methylated[28]. This is an interesting proposal but mtDNA psuedo genes in nDNA are not guaranteed to be methylated, calling into question the efficiency of this method.

All PCR-based methods lead to errors in the analysis of heteroplasmy, due to biases introduced by the variability in PCR efficiencies. In addition, errors arise

bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

from the inability to distinguish clonal reads in PCR amplicons, which can arise from several steps in the preparation of samples for deep sequencing. Additionally, Numts, which exhibit variations and occur in variable copy numbers, confound results in unpredictable ways, both for capture and PCR based methods.

We present here Mito-seek, a novel method to efficiently purify and inexpensively sequence mtDNA. By applying Mito-seek to several cell-lines and human peripheral blood mononuclear cells (PBMC), we identified multiple mtDNA haplotypes in the samples. A major benefit of this method is the ability to call extremely rare variants, the calls are only limited by the sequencing depth. Sequencing errors can also be overcome with depth of sequencing, which is not always possible, especially in PCR amplicon sequencing. Additionally, through clonal expansion of single cells from a variety of cell lines, we establish that heteroplasmy is stably maintained at a single cell level through multiple divisions. This suggests active intercellular exchange of mtDNA. This exchange is explicitly demonstrated by co-culturing two different cell-lines with distinct mtDNA haplotypes, labeling one cell line with GFP, and sorting the cells after twenty passages to show the mtDNA haplotypes unique to one cell-line selectively appear in the other. These results, in conjunction with simulations, suggest the exchange of mtDNA between cells is a source of renewal and stability.

Results and Discussion

Mito-seek: An efficient method to isolate and sequence mtDNA

The potential relevance of mtDNA to many diseases requires a method to accurately determine the diversity of mtDNA in populations of cells. However, as noted, one of the major problem of existing approaches is the presence of nuclear DNA, which contains sequences of high homology to mtDNA (Numts), making it difficult to discern mtDNA from nDNA (Fig. 2). To address this issue, we hypothesized that we could take advantage of the difference in topology between nDNA and mtDNA using an exonuclease to digest the linear nDNA, while leaving intact the circular mtDNA. Total DNA was extracted from HEK 293T cells, and digested with exonuclease V or left undigested. To determine the outcome, we PCR amplified sequences specific to nDNA or mtDNA using appropriate primers. As expected, in the undigested samples of total DNA we could detect both nDNA and mtDNA (Fig.3A, the pcr product lengths are in Fig. 3. In sharp contrast, in the samples treated with exonuclease V we could only detect mtDNA (Fig. 3B).

Next, mtDNA was prepared and sequenced on the Illumina MiSeq platform. Out of a total of 3.05 million 100nt reads, 1.233 million mapped to the mitochondrial genome and 50,000 ($< 2\%$) mapped to the nDNA. The remainder were adapter dimers, which are sequencing artifacts currently filtered out experimentally using Ampure beads. Over 98% of the mappable reads were derived from mtDNA with an average coverage > 3000X (Fig. 3D). More than 50 distinct samples were processed similarly to consistently obtain high purity mtDNA sequence.

The error rate per base of the reads is approximaely 1 in 1000 (Q score > 30). We use a coverage of at least 10 non-clonal reads to make a variant call which reduces the mistakes in variant calling due to sequencing errors to much less than 1 in a million. This also allows removal of variants with a significant bias towards one strand, which is a known source of errors in variant calling from sequencing on

bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

the Illumina platform[29]. Contamination from the small amount of nDNA left in the samples does not contribute appreciably to the noise, due to the small fraction of Numts relative to the total amount of nDNA. Thus, calling rare variants to any level of sensitivity only depends on the depth of sequencing.

This approach, dubbed Mito-seek, provides a means of unmatched efficiency in accurately sequencing the mtDNA contained within a population of cells (Fig. 4).

Ubiquity of heteroplasmy

We expected homoplasmy in tumor cell lines because they are clonal colonies; either a slight fitness advantage of one haplotype will lead to a clonal selection or drift can also lead to homoplasmy[15]. In order to study the diversity of mtDNA in cell lines, we applied Mito-seek to thirty samples including four human PBMCs and human cell lines derived from human diploid fibroblasts (501T), glioma (A382) and breast carcinoma (HCC1806 and MDA-MB-157). This is one of the most detailed and extensive survey of mtDNA from cell lines yet obtained.

The mtDNA sequences were analyzed for mutations, deletions, and duplications, to infer universal features in mtDNA variability and differences between human cell lines and blood-derived mtDNA. Repeat content of the sequences was computationally identified to estimate nDNA contamination, which ranged from $0.5 - 1.5\%$; further confirming the specificity of Mito-seek. Importantly, because of this high degree of mtDNA purity ($> 98\%$) we were able to multiplex all 30 samples in a single MiSeq run, with average coverage of > 100X.

Homoplasmic mtDNA variants should occur at frequencies of either 0 or 1, intermediate values imply the co-existence of more than one haplotype in the population. Strikingly, in both cell-lines and human blood-derived mtDNA, we observed variants occurring in the $0.1 - 0.9$ frequency range (Fig. 5), indicating that multiple haplotypes were present in the samples. The tool *Mutation Assessor*[30] was used to label the mutations as *high*, *medium*, *neutral*, signifying the impact of mutations on protein function. The cell-lines and human PBMCs did not exhibit deleterious mutations at high frequency.

The mtDNA has a few non-coding regions outside of the D-loop which occur as gaps between genes. None of the samples exhibited mutations in these regions, suggesting an evolutionarily conserved role, such as transcriptional control, for these regions. Each sample had unique, distinguishing mutations, ranging in frequency from 0.36 to 1.0. Each of the four human samples have variants that characterize them uniquely (ranging in number from 5 to 15), and the cell lines also have sample-specific variants (5 to 21 unique markers) per cell-line.

Since the cell lines were derived from a variety of tissues, our findings have some level of universality. There were no key distinguishing features between cell-line and human blood-derived mtDNA, in terms of deleterious mutations or degree of heteroplasmy, contrary to findings from a study based on whole-genome sequencing of TCGA samples[25]. Our data is consistent with another study based on colorectal cancer[11].

Stability of heteroplasmy in cell-lines

Though our results indicate there is heteroplasmy (variant frequencies between 0 and 1) in the population of cells, they do not imply heteroplasmy in individual cells,

bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

since a mixture of homoplasmic cells with different haplotypes will give the same result. In order to establish heteroplasmy in individual cells, we placed the severest possible bottleneck on the population by deriving colonies starting from single cells, utilizing MDA-MB-157 and U20S breast carcinoma and osteosarcoma lines respectively (Fig. 6). In all the derived colonies (8 colonies), the variants from the original lines remained in the derived colonies and had approximately the same frequencies as in the original tumor lines. The sharing of mutations between the original and derived colonies suggests that the diversity in mtDNA exists in individual cells. The preservation of the frequencies between the original and derived colonies indicates that the heteroplasmy is uniform across cells in the original line (Fig. 6). Since the new clonal lines underwent at least 25 divisions from the single-cell stage, these results also suggest that heteroplasmy is stably maintained over multiple generations with no signs of selection or drift.

Over so many divisions we would have expected small differences in fitness and drift to lead to homoplasmy. In fact, drift has been proposed as a mechanism for the selection of homoplasmic mtDNA mutations in tumors[15], which has been corroborated in other studies[11]. In light of this, our findings are quite suprising.

A simple model of mtDNA genetics that assumes random assortment of mtDNA haplotypes between daughter cells upon cell division, followed by multiplication of mitochondria, would lead to drift towards homoplasmy, as seen in our simulation of this process (Fig. 7). The rate of drift in haplotype frequencies is a function of the number of mtDNA per cell and the frequency of the haplotypes (Fig. 7). After many passages, irrespective of the original mtDNA distribution, the likelihood of two randomly selected cells having the same heteroplasmic mix is extremely low, which is at odds with the stable and uniform heteroplasmy that we observed in the clonally-derived cell-lines. This suggests the existence of an active mechanism to counteract this drift.

Exchange of mtDNA between cells in a population is the simplest explanation for the uniformity of heteroplasmy and its stability. Exchange can counteract the effects of drift by bringing the haplotype distribution closer to the average of the distribution across cells within the population. Other explanations, such as a balancing selection[31] can be invoked to explain the lack of drift, but they can be discounted because most variants are neutral, while the selection needs to be specific for each cell line, in order to serve as a universal explanation.

Experimental demonstration of mtDNA exchange between cells

In order to explicitly demonstrate the exchange of mtDNA between cells, cells from different cell-lines with distinct private haplotypes were co-cultured. The pair MDA-MB-157 and HCC and a second pair consisting of A382 and U20S were used. For each pair, one of the cell-lines was labeled with GFP (by transfection with a vector expressing GFP). After approximately 20 passages, the cells were sorted using the GFP marker by FACS and the mtDNA from the sorted cells were sequenced. The sorted cells were greater than 99% pure based on the FACS.

Table 1 shows the results of sequencing mtDNA from these co-culturing experiments. Variants private to one cell-line were detected in the co-cultured partner cell-line, suggesting there is transfer of mtDNA between the cell-lines. Not every

bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

private variant gets transferred, arguing against the results arising from errors in sorting or cytoplasmic/nuclear exchange between cells. The purity of the sorted cells, based on FACS analysis, further suggests that nuclear exchange does not occur often (< 1 % contamination).

This is the first explicit demonstration of mtDNA transfer between cells with healthy mtDNA whereas previous studies have shown transfer from cells with healthy mtDNA into ones with non-functioning mtDNA[32, 33].

The selective transfer of mtDNA haplotypes between dissimilar cells could arise from incompatibility between mtDNA haplotypes or between mtDNA haplotypes and the nDNA. Several reports have suggested a link between the mitochondrial and nuclear genotypes and a co-evolution of the two[34]. The selective advantage of certain mtDNA haplotypes can additionally contribute to the stability of the mtDNA.

Discussion

The ability of Numts to confound analyses is highlighted by a study based on whole-genome data from the TCGA, which inferred that deleterious mtDNA mutations are more common in cancer cells compared to normal tissue[25], in contrast to the findings of low mutations rates in tumor mtDNA from a study on colorectal cancer[11] which is more in line with our finding that cell lines don't exhibit higher rates of deleterious mutations compared to cells from humans. Thus, Mito-seek opens up various areas of investigation that were hitherto inaccessible due to the difficulty in obtaining pure mtDNA. By allowing purification of mtDNA to high levels ($\approx 98\%$), accurate determination of heteroplasmy is made feasible and inexpensive, which enables large-scale applications such as mtDNA genotyping of populations and clinical monitoring of somatic mutations in mtDNA. Using Mito-seek, we have established,

- Heteroplasmy is ubiquitous and stable, in every case we studied in primary human lymphocytes and cell-lines
- mtDNA from transformed human cell-lines and primary human lymphocytes are similar with respect to the distributions of densities and frequencies of mutations.
- Non-coding gaps between genes on the mtDNA are highly conserved, indicating potential roles in functions such as replication.
- Clonal amplification of cells does not lead to a selection of particular mtDNA haplotypes.

Our finding that heteroplasmy is widespread is in accord with results from several studies[11, 12]. The stability of mtDNA heteroplasmy is surprising in light of the prevailing belief that mtDNA has higher rates of mutation[34] and the idea that drift can lead to homoplasmy in about 70 generations[15, 11].

The stability of heteroplasmy against drift could arise from exchanges of mtDNA between cells which can be inferred from our cell-line data (Fig. 6) in conjunction with simulations (Fig. 7) and co-culturing experiments (Table 1). The transfer of mtDNA seems to occur in a selective manner, suggesting that either imcompatibilities between the mtDNA haplotypes or incompatibilities between certain haplotypes and the nuclear genome which has, in fact, been suggested before[34]. This is also consistent with a study in mice seems to suggest that mitochondria from different species cannot co-exist[13].

bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Exchange of mtDNA between mitochondria within a cell has been shown to be necessary; knocking out *fusin*, which mediates fusion between mitochondria[35], causes muscles to atrophy through the accumulation of deleterious mutations. *In vivo* exchanges of mitochondria between cells has been demonstrated, in rejuvenation of cells with damaged mitochondria by transfer of healthy mitochondria from mesenchymal stem cells[33]. Rejuventation of cells containing damaged mtDNA by transfer of healthy mtDNA from neighboring cells in culture has also been observed[32]. Ours is the first demonstration of mtDNA transfer between cells with healthy mtDNA.

The exact mechanisms of mtDNA transfer is unclear. Horizontal transfer of genetic material between species of Yeasts has been shown[36]. There is increasing interest in organelle transfer between cells through microtubule formation[37]. The pursuit of these avenues is beyond the scope of this study.

The proposed exchange of mtDNA between cells can explain its stability over the lifetime of an organism, and over generations, inferred from the relative lack of major age-related disorders originating in the mtDNA and the ability to infer geographic origins of a person from the mtDNA sequence. The stability of mtDNA against deleterious mutations could also be enhanced by a coupling between replication and transcription[38], ensuring the depletion of non-functional mtDNA by inefficiencies in their replication.

The study of mtDNA in cell-lines allows us, for the first time, to understand the nature of mtDNA variability and its maintenance in cell populations. This has implications for the search for somatic mutations that can play a role in various human disorders and in aging. Potentially, such mutations might play an important role when the transfer between cells is impeded and mechanisms involved in mtDNA transfer might be fruitful targets for therapeutic intervention.

Conclusions

Mito-seek is a highly sensitive and specific assay for studying heteroplasmy in mtDNA. Using several cell lines and human leukocytes, we have confirmed that heteroplasmy is ubiquitous and shown that it is stable. Exchange of mtDNA between cells in culture seems to stabilize heteroplasmy against drift. A similar mechanism could explain the relative stability of mtDNA against somatic mutations in humans, allowing information on ancestry to be maintained over generations, even in cell lines. The diversity in mtDNA suggests that mtDNA could be a potential source of unexplained heritability in GWAS studies. By making mtDNA sequencing economical, Mito-seek enables large-scale studies of heteroplasmy for GWAS applications and clinical monitoring of mtDNA in tissues, which will be of great value in prognostics and therapeutic intervention. There is potential for the use of transfer of mtDNA as therapy to treat disorders arising from mtDNA defects. There is great value in surveying large populations for establishing the normal range of heteroplasmy, to facilitate its use in GWAS studies.

Methods

Mito-seek

We have developed a new method of isolating and sequencing mtDNA (Fig. 4). The results section contains details of its performance. Briefly, the method consists of

bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

the following steps, total DNA is isolated from the sample. The nDNA is digested using Exonuclease V. The products are purified using Ampure beads to remove short fragments. Using PCR primers specific to mtDNA and nDNA, the purity of the treated samples is tested (Fig. 3B). Following this, the sample is fragmented using Covaris and end-repaired. Barcoded adapters compatible with the sequencing platform are ligated to the fragments. The universal adapters are used to amplify the library and prepare it for deep sequencing.

Cell Culture

mtDNA was isolated and sequenced from several cell lines including, 293T (a kidney-cancer derived cell line), U2OS and Saos-2 (human osteosarcoma cell lines) and MDA-MB-157 (metastatic human breast cancer cell line).

All cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and 50 U/ml penicillin and streptomycin (Pen/Strep; Invitrogen). Cultures were maintained at 37° C in 5% CO2.

Clonal isolation of tumor cells was performed by serial dilution into 96-well plates and visual examination of wells for single cells, which were then expanded for an additional 28-30 population doublings.

Analyses

Sequences that map to repeat elements (which occur only in the nDNA) allow reliable estimation of the level of nDNA contamination, which ranged from $0.5 - 1.5\%$.

MiST[29], a variant detection tool for whole-exome data, was used to call mtDNA variants. The reference mitochondrial genome has the accession NC_012920 from Genbank. The mtDNA annotations are from MITOMAP13, and SNP annotations are from dbSNP14. The error rate in Miseq and Hiseq reads are approximately 1 in a 1000, so requiring at least 3 non-clonal reads to have the variant to make the call, reduces the error rate to well under 1 in a million. Variants with reads predominantly in one strand are excluded to firther reduce errors, based on our previous experience[29].

We developed a pipeline to assemble the mitochondrial genome from the deep-sequencing data, to demonstrate that the reads assemble into a circle and no large deletions, duplications or other large-scale structures were detected.

Mutation Assessor[30] was used to assess the impact of mtDNA mutations on protein function. This tool uses conservation of structure across orthologues to identify mutations in the DNA (and consequent changes in amino-acids) with potentially deleterious effects. The mutations are rated *high*, *medium*, *low*, or *neutral* based on their impact on protein function. We highlight the *high* and *medium* impact mutations in our graphs, as they may affect mitochondrial function.

Competing interests

Author's contributions

AJ and RS designed Mito-seek and designed several experiments. SA and MW designed several experiments with cell-lines. LL provided human samples and suggested applications. AJ, JS, RL performed Mito-seek, EB performed cell-line work. AJ and RS wrote the paper.

bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Acknowledgements

RS, AJ, LL were partially supported by the grant 1R21HG007394-01 from the NIH. Some experiments were also funded by a pilot grant from the Venture Capital Research Funding Program of Children's Environmental Health Center (CEHC) at Mount Sinai. Brian Brown helped craft the message. Comments from Viviana Simon, and Benjamin tenOever added clarity. Avinash Waghray, Sunniva Bjorklund and Vessela Kristensen caught numerous errors and gave many suggestions to improve the writing.

Author details

[1]Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai , One Gustave L. Levy place, New York, 10029, USA. [2]Department of Public Health, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy place, New York, USA. [3]Cold Spring Harbor Laboratory, One Bungtown Road, Cold Spring Harbor, USA.

References

1. Wallace, D.C.: Structure and evolution of organelle genomes. Microbiological Reviews **46**(2), 208–240 (1982). PMID: 6750346
2. Wallace, D.C.: Mitochondrial diseases in man and mouse. Science (New York, N.Y.) **283**(5407), 1482–1488 (1999). PMID: 10066162
3. Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X.: Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell **86**(1), 147–157 (1996). PMID: 8689682
4. Newmeyer, D.D., Ferguson-Miller, S.: Mitochondria: releasing power for life and unleashing the machineries of death. Cell **112**(4), 481–490 (2003). PMID: 12600312
5. Westermann, B., Neupert, W.: 'Omics' of the mitochondrion. Nature biotechnology **21**(3), 239–240 (2003). PMID: 12610566
6. Brandon, M.C., Lott, M.T., Nguyen, K.C., Spolim, S., Navathe, S.B., Baldi, P., Wallace, D.C.: MITOMAP: a human mitochondrial genome database–2004 update. Nucleic Acids Research **33**(Database issue), 611–613 (2005). PMID: 15608272
7. Jukes, T.H., Osawa, S.: The genetic code in mitochondria and chloroplasts. Experientia **46**(11-12), 1117–1126 (1990). Accessed 2014-07-08
8. Legros, F., Malka, F., Frachon, P., Lombes, A., Rojo, M.: Organization and dynamics of human mitochondrial DNA. Journal of cell science **117**(Pt 13), 2653–2662 (2004)
9. Fernandez-Vizarra, E., Enriquez, J.A., Perez-Martos, A., Montoya, J., Fernandez-Silva, P.: Tissue-specific differences in mitochondrial activity and biogenesis. Mitochondrion **11**(1), 207–213 (2011). Accessed 2013-04-25
10. Wallace, D.C.: Mitochondrial DNA mutations in diseases of energy metabolism. Journal of bioenergetics and biomembranes **26**(3), 241–250 (1994). PMID: 8077179
11. He, Y., Wu, J., Dressman, D.C., Iacobuzio-Donahue, C., Markowitz, S.D., Velculescu, V.E., Diaz, J. Luis A, Kinzler, K.W., Vogelstein, B., Papadopoulos, N.: Heteroplasmic mitochondrial DNA mutations in normal and tumour cells. Nature **464**(7288), 610–614 (2010). PMID: 20200521
12. Diroma, M.A., Calabrese, C., Simone, D., Santorsola, M., Calabrese, F.M., Gasparre, G., Attimonelli, M.: Extraction and annotation of human mitochondrial genomes from 1000 genomes whole exome sequencing data. BMC Genomics **15**(Suppl 3), 2 (2014). Accessed 2014-07-07
13. Sharpley, M.S., Marciniak, C., Eckel-Mahan, K., McManus, M., Crimi, M., Waymire, K., Lin, C.S., Masubuchi, S., Friend, N., Koike, M., Chalkia, D., MacGregor, G., Sassone-Corsi, P., Wallace, D.C.: Heteroplasmy of mouse mtDNA is genetically unstable and results in altered behavior and cognition. Cell **151**(2), 333–343 (2012). Accessed 2013-02-19
14. Jokinen, R., Marttinen, P., Sandell, H.K., Manninen, T., Teerenhovi, H., Wai, T., Teoli, D., Loredo-Osti, J.C., Shoubridge, E.A., Battersby, B.J.: Gimap3 regulates tissue-specific mitochondrial DNA segregation. PLoS Genet **6**(10), 1001161 (2010). Accessed 2013-02-19
15. Coller, H.A., Khrapko, K., Bodyak, N.D., Nekhaeva, E., Herrero-Jimenez, P., Thilly, W.G.: High frequency of homoplasmic mitochondrial DNA mutations in human tumors can be explained without selection. Nature Genetics **28**(2), 147–150 (2001)
16. Wallace, D.C., Chalkia, D.: Mitochondrial DNA genetics and the heteroplasmy conundrum in evolution and disease. Cold Spring Harbor Perspectives in Biology **5**(11), 021220 (2013)
17. Lightowlers, R.N., Chinnery, P.F., Turnbull, D.M., Howell, N.: Mammalian mitochondrial genetics: heredity, heteroplasmy and disease. Trends in genetics: TIG **13**(11), 450–455 (1997). PMID: 9385842
18. Cree, L.M., Samuels, D.C., de Sousa Lopes, S.C., Rajasimha, H.K., Wonnapinij, P., Mann, J.R., Dahl, H.-H.M., Chinnery, P.F.: A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. Nature Genetics **40**(2), 249–254 (2008). Accessed 2013-04-22
19. Woischnik, M., Moraes, C.T.: Pattern of organization of human mitochondrial pseudogenes in the nuclear genome. Genome Research **12**(6), 885–893 (2002). PMID: 12045142 PMCID: PMC1383742. Accessed 2013-04-25
20. Maricic, T., Whitten, M., Paabo, S.: Multiplexed DNA sequence capture of mitochondrial genomes using PCR products. PLoS ONE **5**(11), 14004 (2010). Accessed 2013-04-25
21. Paull, D., Emmanuele, V., Weiss, K.A., Treff, N., Stewart, L., Hua, H., Zimmer, M., Kahler, D.J., Goland, R.S., Noggle, S.A., Prosser, R., Hirano, M., Sauer, M.V., Egli, D.: Nuclear genome transfer in human oocytes eliminates mitochondrial DNA variants. Nature **advance online publication** (2012). Accessed 2013-04-25
22. Vallone, P.M.: Capillary electrophoresis of an 11-plex mtDNA coding region SNP single base extension assay for discrimination of the most common caucasian HV1/HV2 mitotype. Methods in molecular biology (Clifton, N.J.) **830**, 159–167 (2012). PMID: 22139659
23. Ameur, A., Stewart, J.B., Freyer, C., Hagstrom, E., Ingman, M., Larsson, N.-G., Gyllensten, U.: Ultra-deep sequencing of mouse mitochondrial DNA: mutational patterns and their origins. PLoS Genet **7**(3), 1002028 (2011). Accessed 2013-02-19

bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

24. Sosa, M.X., Sivakumar, I.K.A., Maragh, S., Veeramachaneni, V., Hariharan, R., Parulekar, M., Fredrikson, K.M., Harkins, T.T., Lin, J., Feldman, A.B., Tata, P., Ehret, G.B., Chakravarti, A.: Next-generation sequencing of human mitochondrial reference genomes uncovers high heteroplasmy frequency. PLoS computational biology **8**(10), 1002737 (2012). PMID: 23133345
25. Larman, T.C., DePalma, S.R., Hadjipanayis, A.G., Protopopov, A., Zhang, J., Gabriel, S.B., Chin, L., Seidman, C.E., Kucherlapati, R., Seidman, J.G.: Spectrum of somatic mitochondrial mutations in five cancers. Proceedings of the National Academy of Sciences **109**(35), 14087–14091 (2012). Accessed 2013-02-19
26. Picardi, E., Pesole, G.: Mitochondrial genomes gleaned from human whole-exome sequencing. Nature methods **9**(6), 523–524 (2012). PMID: 22669646
27. Chu, H.-T., Hsiao, W.W.L., Tsao, T.T.H., Chang, C.-M., Liu, Y.-W., Fan, C.-C., Lin, H., Chang, H.-H., Yeh, T.-J., Chen, J.-C., Huang, D.-M., Chen, C.-C., Kao, C.-Y.: Quantitative assessment of mitochondrial DNA copies from whole genome sequencing. BMC genomics **13 Suppl 7**, 5 (2012). PMID: 23282223
28. McKernan, K.J., Spangler, J., Zhang, L., Tadigotla, V., McLaughlin, S., Warner, J., Zare, A., Boles, R.G.: Expanded genetic codes in next generation sequencing enable decontamination and mitochondrial enrichment. PLoS ONE **9**(5), 96492 (2014). Accessed 2014-06-19
29. Subramanian, S., Pierro, V.D., Shah, H., Jayaprakash, A.D., Weisberger, I., Shim, J., George, A., Gelb, B.D., Sachidanandam, R.: MiST: a new approach to variant detection in deep sequencing datasets. Nucleic Acids Research (2013). PMID: 23828039. Accessed 2013-07-05
30. Reva, B., Antipin, Y., Sander, C.: Predicting the functional impact of protein mutations: application to cancer genomics. Nucleic Acids Research (2011). Accessed 2013-02-19
31. Connallon, T., Clark, A.G.: Balancing selection in species with separate sexes: Insights from fisher's geometric model. Genetics (2014)
32. Spees, J.L., Olson, S.D., Whitney, M.J., Prockop, D.J.: Mitochondrial transfer between cells can rescue aerobic respiration. Proceedings of the National Academy of Sciences of the United States of America **103**(5), 1283–1288 (2006). Accessed 2014-07-08
33. Islam, M.N., Das, S.R., Emin, M.T., Wei, M., Sun, L., Westphalen, K., Rowlands, D.J., Quadri, S.K., Bhattacharya, S., Bhattacharya, J.: Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. Nature medicine **18**(5), 759–765 (2012). PMID: 22504485
34. Dowling, D.K., Friberg, U., Lindell, J.: Evolutionary implications of non-neutral mitochondrial genetic variation. Trends in Ecology & Evolution **23**(10), 546–554 (2008). Accessed 2014-07-07
35. Chen, H., Vermulst, M., Wang, Y.E., Chomyn, A., Prolla, T.A., McCaffery, J.M., Chan, D.C.: Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. Cell **141**(2), 280–289 (2010). PMID: 20403324
36. Marinoni, G., Manuel, M., Petersen, R.F., Hvidtfeldt, J., Sulo, P., Piškur, J.: Horizontal transfer of genetic material amongSaccharomyces yeasts. Journal of Bacteriology **181**(20), 6488–6496 (1999). PMID: 10515941. Accessed 2014-01-04
37. Rogers, R.S., Bhattacharya, J.: When cells become organelle donors. Physiology (Bethesda, Md.) **28**(6), 414–422 (2013). PMID: 24186936
38. Clayton, D.A.: Transcription and replication of mitochondrial DNA. Human reproduction (Oxford, England) **15 Suppl 2**, 11–17 (2000). PMID: 11041509

Figures

Tables

bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure 1 Organization of mtDNA. 13 protein-coding genes, 22 tRNA genes and 2 rRNA genes are encoded on a single circular nucleic acid and transcribed from three promoters: LSP (inner circle of genes - strand), HSP1 (outer circle of gene, + strand) and HSP2 (16S rRNA) on the D-loop, which is non-coding but critical for replication and transcription. The three polycistronic transcripts are processed by enzymatic excision of the tRNAs. There are a few small gaps (< 30 nt) in annotation, and a 45nt overlap between ATP6 and ATP8 which might have roles in replication.

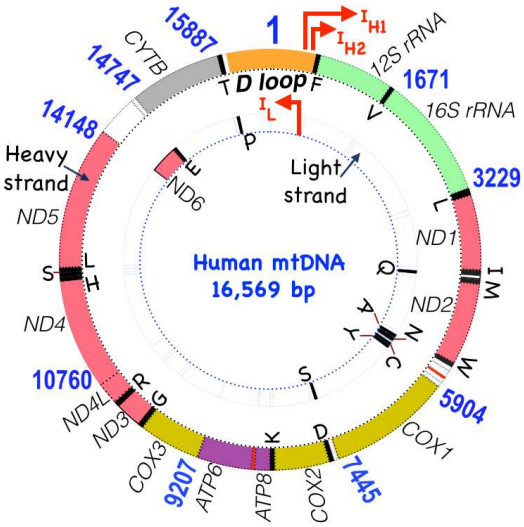
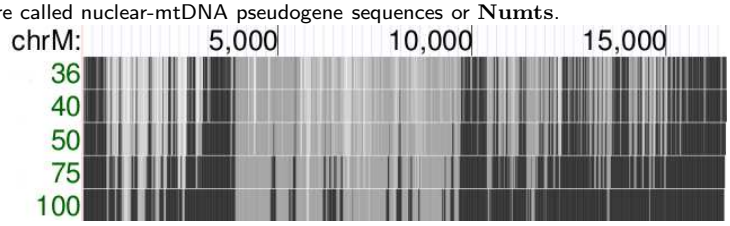
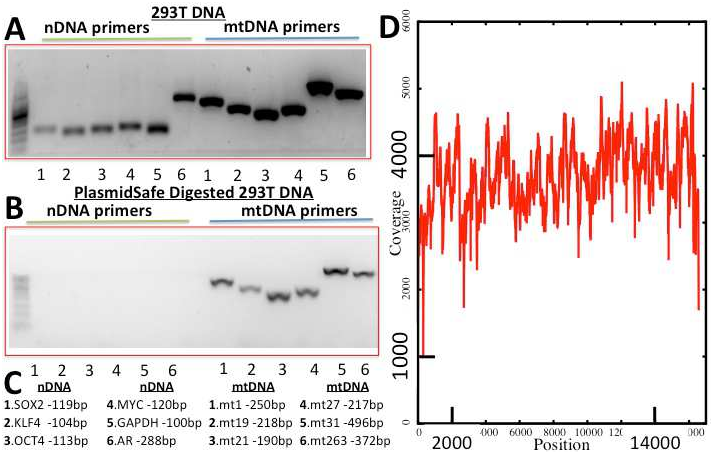


Figure 2 Mappings of mtDNA tiles. Each band shows mappings of n-mer tiles ($n = 36, 40, 50, 75, 100$) from mtDNA on the human genome. Darker regions are more unique. Even 100 nt tiles from mtDNA often map to nDNA; thus, unambiguous identification of mtDNA variants requires efficient isolation of mtDNA. The pseudo-gene copies of mtDNA in the nuclear genome are called nuclear-mtDNA pseudogene sequences or **Numts**.



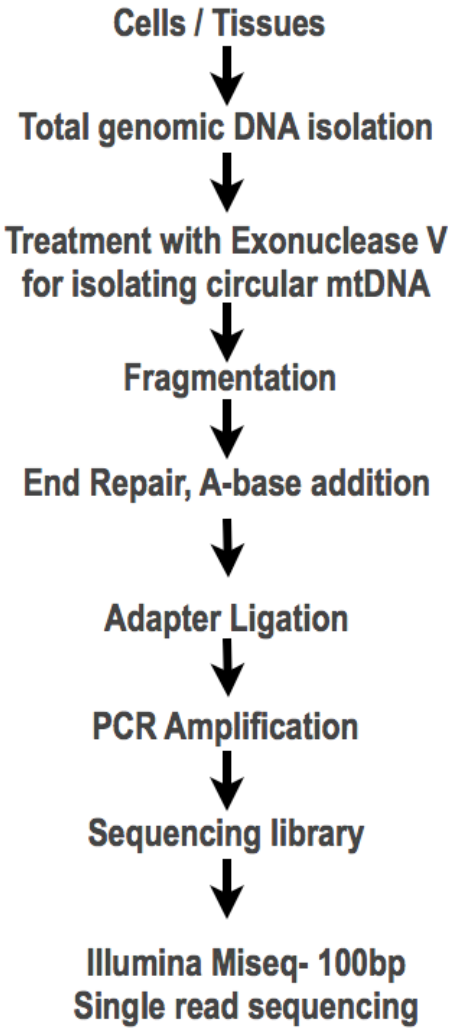
bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure 3 Performance of Mito-seek. (**A**) PCR products run on a 2% Agarose gel using primers for 6 nDNA genes (OCT4, MUC, KLF4, SOX2, GAPDH and AR) and 5 regions of mtDNA. before exonuclease digestion. (**B**) After digestion, the nDNA bands disappear. (**C**) Sizes of expected PCR products. (**D**) Deep sequencing, read depth (y-axis) versus position on mtDNA (x-axis). 1.23 million mtDNA reads and 50,000 nDNA reads implying $> 98\%$ pure mtDNA.



bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure 4 The Mito-seek protocol.



bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure 5 Novel variants in a cell-line (MDA-MB-157, circles) and a human (PBMC, crosses). Mutation frequency (y-axis) versus position on mtDNA (x-axis). Genes are colored bands at bottom of graph ($+, -$ represent the strand, cds is coding sequence). *Neutral*, *Low*, *Medium*, and *High* are the effect of the mutation on protein function (*Mutation Assessor*[30]). Except for the D-loop, most of the mtDNA codes for a transcript, with a few gaps. The longer gaps (11, 24, and 30 nt long) are marked by vertical red lines. The 45 nt long overlapping region between ATP8 and ATP6 is marked by black vertical lines. Mutation frequencies between 0 and 1 arise from the co-existence of multiple mtDNA haplotypes. Heteroplasmy at a cellular level is demonstrated in Fig. 6. There are no stark differences between the human and cell-line derived mtDNA.

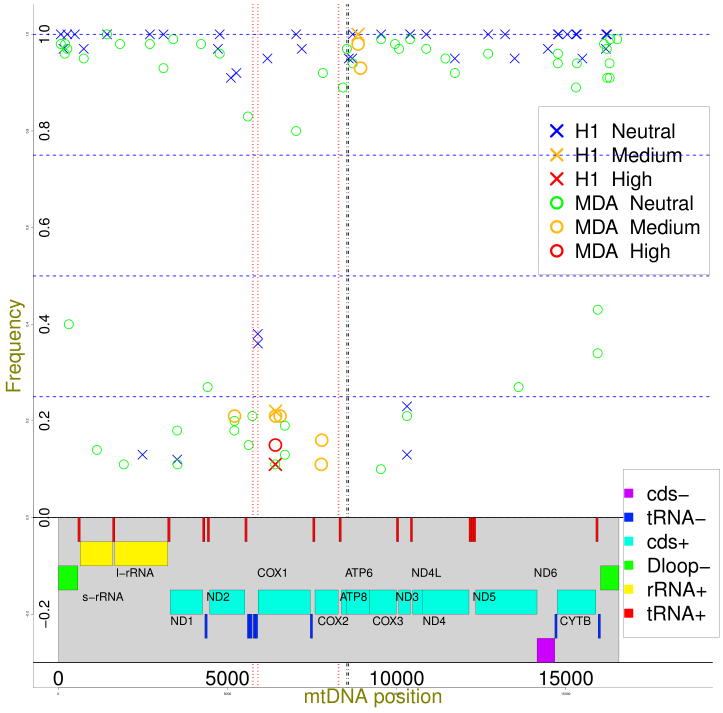
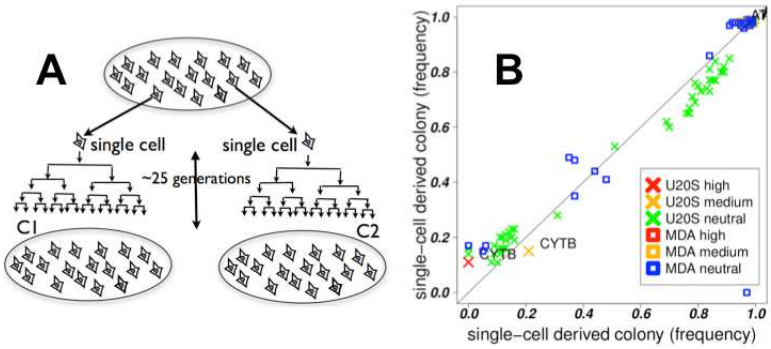
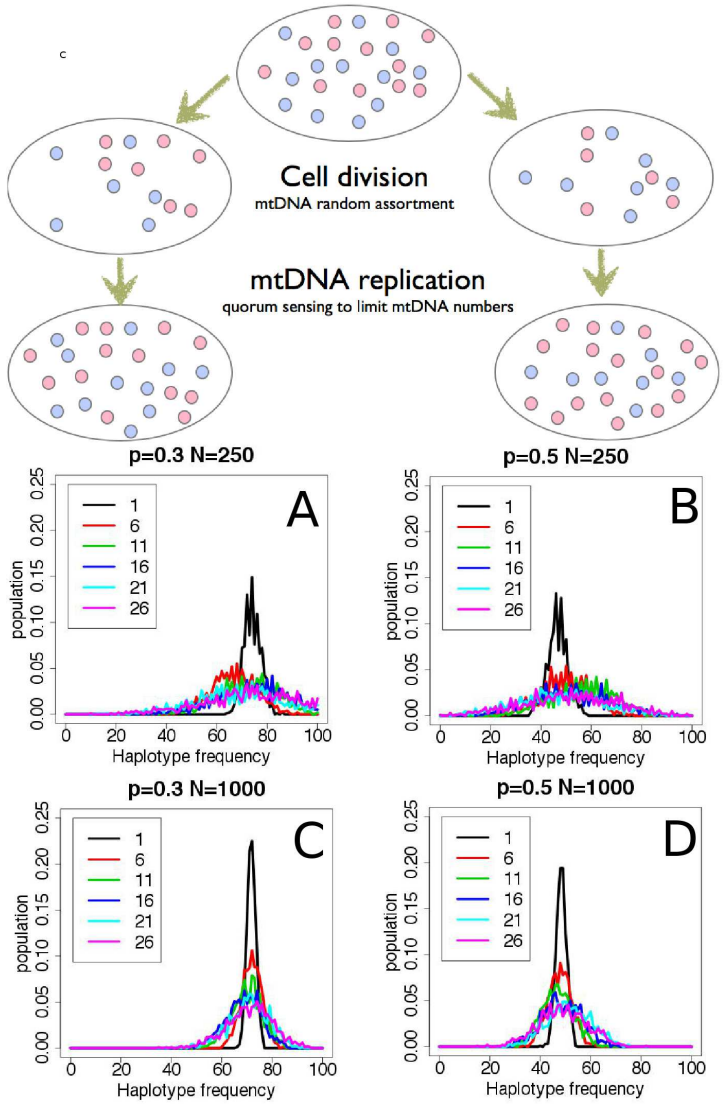


Figure 6 Stability of heteroplasmy. **A** C1 and C2 are colonies derived from single cells in the original colony, placing a severe botteneck on the mtDNA, and then passaged ≈ 25 times. **B** Mutation frequencies in C1 (x-axis) versus C2 (y-axis) for two cell-lines, U20S and MDA-MB-157. The mutations mostly lie along the diagonal; the heteroplasmic mix of mutations in the derived colonies are similar to each other. This implies the heteroplasmic mix exists at the single-cell level, and is stable over many divisions. A drift in frequencies is expected with random assortment of mtDNA haplotypes (simulations, Fig. 7). The stability of the frequencies implies active mechanisms to counteract the drift, such as the exchange of mtDNA between cells.



bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure 7 Simulations of mtDNA replication. Each cell contains a mixture of mtDNA haplotypes, here we consider only two species, red and blue, for the sake of simplicity. At cell division, the mitochondria assort randomly between the daughter cells and divide (with mtDNA replication) till a quorum of mitochondria, specific to each tissue-type, is reached. Quorum sensing is not well-understood, but the number of mitochondria per cell is tissue-specific[9]. The graphs show simulations of the evolution of heteroplasmy over time, based on this model. The distribution of haplotype frequencies spreads over time (drift), implying two randomly selected cells are unlikely to have the same mtDNA haplotype distribution. The plots show the distribution of frequencies after 1, 6, 11, 16, 21 and 26 divisions. The starting number of mitochondria per cell (N) is 250 in the upper panels (A,B) and 1000 in the lower panels (C,D). One of the alleles occurs with a frequency (p) of 0.3 in the left panels (A,C) and p=0.5 in the right panels (B,D). The drift is slower and the distributions narrower with larger N and smaller deviations of p from 0.5.



bioRxiv preprint doi: https://doi.org/10.1101/007005; this version posted July 11, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Table 1 Mixing experiments. Data from 1) HCC co-cultured with MDA-MB-157, and 2) U20S co-cultured with A382 are depicted here. The column private_to identifies the cell-lines that exhibit the variant. The dark gray highlights rows where a variant unique ot A382 has been identified in U20S cells co-cultured with A382, while the light gray highlights cases where a variant unique to HCC has been identified in MDA-MB-157 cells co-cultured with HCC. The light blue rows are variants private to A382 that did not transfer into U20S. The light green rows are variants private to HCC that did not transfer into MDA-MB-157. For example, position 3796 (row 7), the A from the reference mtDNA genome is mutated to a T only in HCC, the U20S cultured with A382 exhibits only an A, but the MDA-MB-157 cultured with HCC exhibits an A, with a frequency of 0.14 (or 14%). f is the frequency and c is the coverage.

mut	gene	AA	private_to	HCC in MDA-MB-157(f|c)	MDA-MB-157 in HCC(f|c)	U20S in A382(f|c)	flank
T146C	D-loop		U20S	0|11	0|5	0.97|37	ATTCCTGCCT[T|c]ATCCTATTAT
C315CC	D-loop		A382	0|1	0|4	0.23|30	CCCCTCCCCC[C|Cc]CTTCTGGCCA
G3010A	l-rRNA		U20S	0|33	0|23	1|87	AGGACATCCC[G|a]ATGGTGCAGC
G3666A	ND1	G120G	HCC	0.93|33	0|25	0|87	TCTGATCAGG[G|a]TGAGCATCAA
C3699G	ND1	G131G	U20S	0|27	0.07|26	0.98|85	CCCTGATCGG[C|g]GCACTGCGAG
A3796T	ND1	T163S	HCC	0.88|9	0.14|21	0|110	TAACCTCTCC[A|t]CCCTTATCAC
A4104G	ND1	L266L	HCC	0.9|21	0.06|31	0|165	TTCTAACCTC[A|g]CTGTTCTTAT
T4216C	ND1	Y303H	U20S	0|3	0|10	1|64	ACTTATATGA[T|c]ATGTCTCCAT
C7819A	COX2	L78L	MDA-MB-157	0|35	0.76|43	0|138	CGCATCCTTT[C|a]CATAACAGAC
T7861C	COX2	D92D	HCC	0.96|29	0.05|39	0|140	CGGACTAATC[T|c]TCAACTCCTA
A8701G	ATP6	T58A	A382|MDA-MB-157|HCC	0|0	0.85|14	0|121	ACAAATGATA[A|g]CCATACACAA
C8932T	ATP6	P135S	MDA-MB-157	0|25	0.9|21	0|131	CCATACTAGT[C|t]ATTATCGAAA
G9064A	ATP6	A179T	HCC	1|10	0.16|24	0|125	CCATTAACCT[G|a]CCCTCTACAC
A9072G	ATP6	S182S	HCC	0.88|9	0.18|22	0|124	CTTCCCTCTA[A|g]ACTTATCATC
C9212T	COX3	T2T		0|29	0.2|39	0|147	CATGCCTATC[C|t]TATAGTAAAA
T9540C	COX3	L111L	A382|MDA-MB-157|HCC	0.86|15	1|11	0|65	TACCCCCCAA[T|c]TAGGAGGGCA
A9545G	COX3	G113G	HCC	0.93|15	0|13	0|74	CCCAATTAGG[A|g]GGGCACTGGC
A10398G	ND3	T113A	HCC|MDA-MB-157|U20S	0|0	1|2	1|35	ATTAGACTGA[A|g]CCGAATTGGT
G10685A	ND4L	A72A	U20S	0|26	0.1|20	1|98	GCGAAGCAGC[G|a]GTGGGCCTAG
G10688A	ND4L	V73V	HCC	0.96|26	0.1|19	0|109	ACCTGACTCC[G|a]ACCCCTCACA
A11251G	ND4	L164L	U20S	0.05|18	0|28	1|127	TCATCGCACT[A|g]ATTTACACTC
A12612G	ND5	V92V	U20S	0|6	0|8	1|62	TCATCCCTGT[A|g]GCATTGTTCG
A12810G	ND5	W158W	HCC	0.92|25	0|20	0|84	AATCCTATAC[A|g]ACCGTATCGG
T13281C	ND5	V315V	U20S	0.14|14	0|35	0.99|127	TCATAATAGT[T|c]ACAATCGGCA
T13602C	ND5	Y422Y	MDA-MB-157	0 |22	0.45|24	0|136	CAAGCGCCTA[T|c]AGCACTCGAA
G13708A	ND5	A457T	U20S	0|9	0.125|16	0.98|93	TAAACGCCTG[G|a]CAGCCGGAAG
A13933G	ND5	T532A	U20S	0|17	0|31	0.99|115	CCCTAGCATC[A|g]CACACCGCAC
T14798C	CYTB	F17L	U20S	0|4	0|15	1|61	TAACCACTCA[T|c]TCATCGACCT
C14911T	CYTB	Y55Y	HCC	0.96|29	0|39	0|105	CTATATTACG[C|t]ATCATTTCTC
C15263A	CYTB	P172T	U20S	0|27	0|27	0.17|129	AGTAGACAGT[C|a]CCACCCTCAC
G15301A	CYTB	L185L	A382|MDA-MB-157	0|19	0.86|22	0|133	ACTTCATCTT[G|a]CCCTTCATTA
C15452A	CYTB	L235I	U20S	0.25|4	0|38	0.97| 135	ACTTCTCTTC[C|a]TTCTCTCCTT
T15514C	CYTB	Y256Y	MDA-MB-157	0 |12	0.89| 28	0|145	CAGACAATTA[T|c]ACCCTAGCCA
C16069T	D-loop		U20S	0|19	0|7	1|78	AGTATTGACT[C|t]ACCCATCAAC
C16108T	D-loop		U20S	0|15	0|6	0.98|71	ACATTACTGC[C|t]AGCCACCATG
T16126C	D-loop		U20S	0 |10	0| 13	1|89	ATGAATATTG[T|c]ACGGTACCAT
C16187T	D-loop		HCC	1|9	0|31	0|143	ATCAAAACCC[C|t]CTCCCCATGC
T16209C	D-loop		MDA-MB-157	0|17	0.92| 26	0|157	TACAAGCAAG[T|c]ACAGCAATCA
C16223T	D-loop		A382|MDA-MB-157|HCC	0.95| 20	0.96| 31	0| 168	GCAATCAACC[C|t]TCAACTATCA
C16292T	D-loop		MDA-MB-157	0|15	0.76| 21	0|175	CAAACCTACC[C|t]ACCCTTAACA
C16295T	D-loop		MDA-MB-157	0|14	0.72| 22	0|174	ACCTACCCAC[C|t]CTTAACAGTA
T16311C	D-loop		HCC|MDA-MB-157	0.75|12	0.8|20	0|150	CAGTACATAG[T|c]ACATAAAGCC