Supplemental Materials:

Inventory of Supplement:

Supplemental Figure 1. Sample lineage tree depicting the relationship between samples. This figure shows the pedigree of all of the samples processed in this study, with Figure 2 in the main text highlighting important relationships between a subset of samples derived from the same pedigree (mother).

Supplemental Figure 2. Sample lineage tree scaled to SNV events along each lineage. This figure shows the mutational burden in each sample in the context of the relationships between samples (pedigree), with Figure 2 in the main text highlighting pedigrees with unusual mutational burden compared to the others.

Supplemental Figure 3. Relationship between mouse or human single mitochondrion samples and allele identities at high confidence positions. This figure shows the mutations present for mother and pup at the high confidence positions identified in both mouse and human, which are characterized further in Figure 2 and Figure 3 in the main text, respectively.

Supplemental Table 1. Primer sequences used for PCR amplification of mouse single mitochondrion genomic samples. This table provides the sequences of primers used to achieve the amplification strategy highlighted in Figure 1.

Supplemental Table 2. Primer sequences used for PCR amplification of human single mitochondrion genomic samples. This table provides the sequences of primers used to achieve the amplification strategy highlighted in Figure 1.

Supplemental Information. Experimental Methods and PCR error model for calling single nucleotide variants. Additional experimental methods used in this work are presented here. Additionally, this supplement provides more detailed mathematical descriptions of how a threshold was set for calling a mutation as a single nucleotide variant.

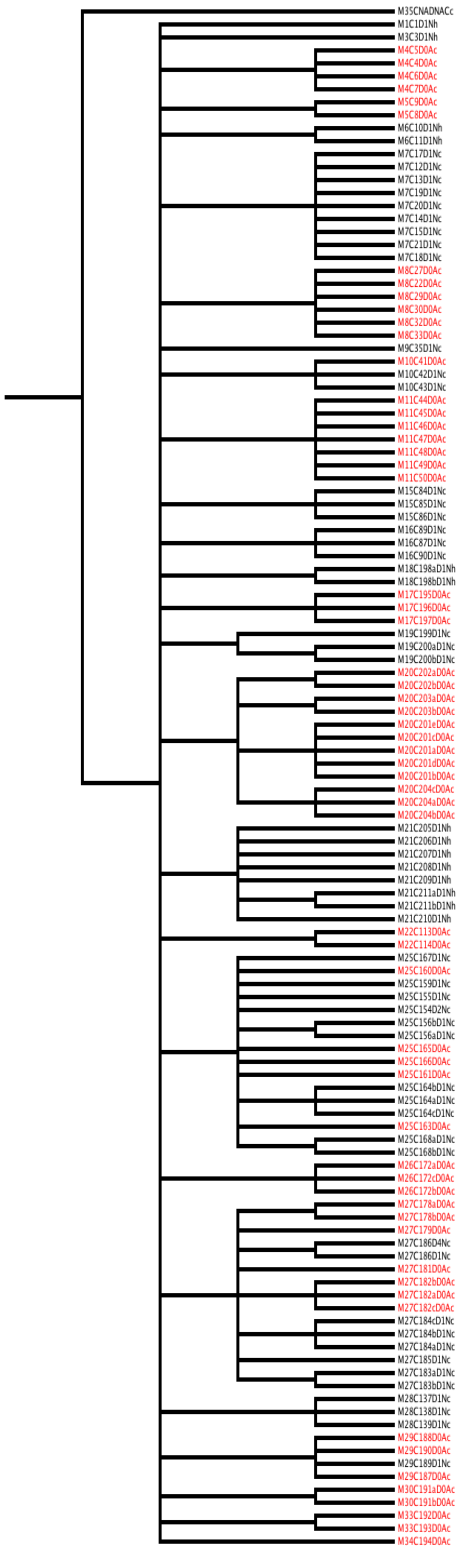

Fig. S1. Sample lineage tree depicting the relationship between samples. Related to Figure 2. Each sample is represented by the generic code MxCxDx[N/A/C][h/c/b] where x is a number. Mx = mother ID. Cx = cell ID. Cx is appended with a lowercase letter for samples isolated from the same cell. Dx = dendrite ID, Dx = 0 for astrocytes. [N/A/C] = cell type, where N=neuron, A=astrocyte, C=population. [h/c/b] = brain region from which the sample was derived: h=hippocampus, c=cortex, b=cerebellum. A reference population sample is grouped by itself on the left of the single mitochondrion samples

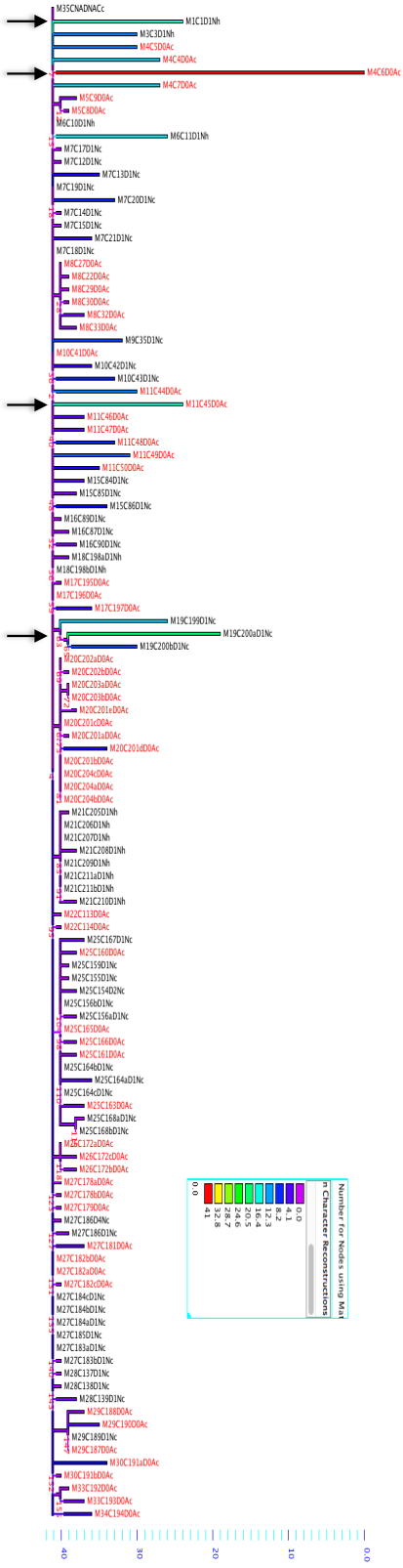

Fig. S2. Sample lineage tree scaled to SNV events along each lineage. Related to Figure 2. The branch lengths of the pictured cell lineage tree are scaled by estimated mutation events along each branch. The Maximum Parsimony method (see Methods) of character reconstruction was used to estimate for each branch the mutation events for the positions with changes in the major allele as well as those positions with changes in the minor allele.

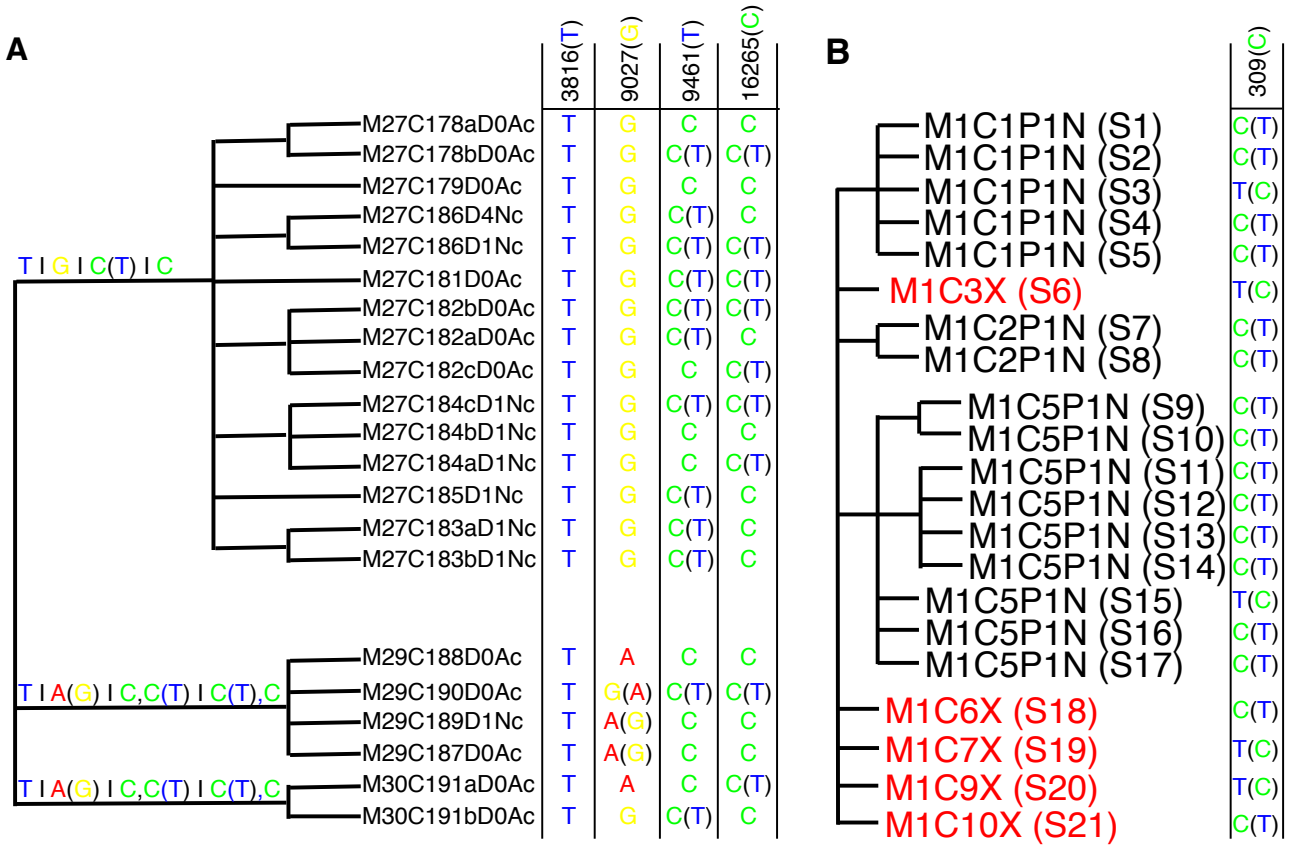

Fig. S3. Relationship between mouse or human single mitochondrion samples and allele identities at high confidence positions. Related to Figure 2 and Figure 3. Sample lineage trees of mouse **(A)** or human (**B**) single mitochondrion with Major(minor) alleles indicated for each high confidence position. The reference allele is indicated in parentheses next to each position label. Major(minor) alleles are listed for mouse mother and pup at the beginning of each branch point for each mother for each position. There is a single entry when the mother and pup alleles agreed. Human sample code format is MxCxPxT where M =mother ID, C = cell ID, P = process ID, T =cell type where N = neuronal-like or X = non-neuronal, with simplified codes in parentheses.

Table S1. Primer sequences used for PCR amplification of mouse single mitochondrion genomic samples. Related to Figure 1.

Version 1 Long PCR Primers

	Forward	Reverse
Amplicon 1	CCATAAACACAAAGGTTTGGTCC	GGTTGACCTAATTCTGCTCGAAT
Amplicon 2	GGAAACTGACTTGTCCCA	GCGTAAGCAGATTGAGCT
Amplicon 3	CGCCTACTCCTCAGTTAGC	AGAGTTTTGGTTCACGGA

Version 2 Long PCR Primers

	Forward	Reverse
Amplicon 1	ATTACAGCTAGAAACCCC	GCCATAGAATAACCCTGG
Amplicon 2	GTCCTAGAAATGGTTCCA	AATGGTATTCCTGTGAGG
Amplicon 3	AGGGTTCTACTCAAAAGA	TAGTCTTTCATCTTTCCC

Short PCR primers

	Forward	Reverse
Set 1	CCATAAACACAAAGGTTTGGTCC	TGTTGAGCTTGAACGCT
Set 2	CCAATTCTCCAGGCATACG	TCCTTCTGTCAGGTCGA
Set 3	CGGCCCATTCGCGTTAT	GGTTGACCTAATTCTGCTCGAAT
Set 4	GGAAACTGACTTGTCCCA	GCGAGATGATATAGAGGACTAAGG
Set 5	CCTTATCATAATTGCTCTCCCC	CGCTCAGAAGAATCCTGCAAAGAA
Set 6	ATCATTCTAGCCTCGTACCA	GCGTAAGCAGATTGAGCT
Set 7	CGCCTACTCCTCAGTTAGC	TTATCAGGCCTAGTTGGC
Set 8	ACCACACCTAGCATTCC	CCGTTTGCGTGTATATATCGG
Set 9	GCTTATTCCTTCATGTCGGAC	AGAGTTTTGGTTCACGGA
Set 10	CCATAAACACAAAGGTTTGGTCC	TAGTCTTTCATCTTTCCCTTGCG
Set 11	ATTACAGCTAGAAACCCCG	AGGTGGCTCTATTTCTCTTG
Set 12	ACGTACACCCTCTAACCTAG	AGGTTGGTGCTGGATATTG
Set 13	ATATGAGTAGGCCTGGAATTCAGC	GGTTGACCTAATTCTGCTCGAAT
Set 14	GGAAACTGACTTGTCCCA	GTTTTGAAGCAAAGGCCTC
Set 15	ATGACTTCATGGCTGCC	CGGTTGTTGATTAGGCGT
Set 16	ACACCAAAAGGACGAACATG	CAGGTTAATTACTCTCTTCTGGG
Set 17	AGTTGCATTCTGACTCCC	GCGTAAGCAGATTGAGCT
Set 18	CGCCTACTCCTCAGTTAGC	ATGTCTCCGATGCGGTT
Set 19	AATCAGCACAATTTGGCC	GTGATCTTTGTTTGCGGGTA
Set 20	CAAAGATCACCCAGCTACTACCAT	GGATTGAGCGTAGAATGGC
Set 21	ACTAGGAGGTGTCCTAGC	AGAGTTTTGGTTCACGGA
Set 22	CCATAAACACAAAGGTTTGGTCC	GCTACCTTTGCACGGTC
Set 23	TGACCTTTCAGTGAAGAGG	TGTCCTAGAAATAAGAGGGCTTG
Set 24	GTGCTACCTAAACACCTTATCC	GGTTGACCTAATTCTGCTCGAAT
Set 25	GGAAACTGACTTGTCCCA	GCCATAGAATAACCCTGGTCG
Set 26	GTCCTAGAAATGGTTCCACT	CCATGGAATCCAGTAGCC
Set 27	GAAGCCGCAGCATGATA	GCGTAAGCAGATTGAGCT
Set 28	CGCCTACTCCTCAGTTAGC	AATGGTATTCCTGTGAGGG
Set 29	AGGGTTCTACTCAAAAGACC	GCTATGACTGCGAACAGT
Set 30	TTGGAACAACCCTAGTCG	AGAGTTTTGGTTCACGGA
Set 31	ATTACAGCTAGAAACCCCG	GTCAGGCTGGCAGAAGTA
Set 32	TTATCTCAACCCTAGCAGAA	GATGGTGGTAGGAGTCAA
Set 33	CGGAAATCTAGCCCATGC	GCCATAGAATAACCCTGGTCG
Set 34	GTCCTAGAAATGGTTCCACT	GGAAAAGTCAGACTACGTC
Set 35	TGAGGATCTTACTCCCTTAGTA	AAGCTTCATGGAGTTTGG
Set 36	GAACGGATCCACAGCCGT	AATGGTATTCCTGTGAGGG
Set 37	AGGGTTCTACTCAAAAGACC	TCCATAATATAAGCCTCGTCC
Set 38	AGAAACCTGAAACATTGGAG	GGGTTTGGCATTAAGAGG
Set 39	ACCCTATGTCCTGATCAA	TAGTCTTTCATCTTTCCCTTGCG
Set 40	ATTACAGCTAGAAACCCCG	AGTTTTTGGGCCATTAGG
Set 41	AACGGACCAAGTTACCCT	GGGTTATTGTGCTTATGATAGC
Set 42	GGACTGTAAGACTTCATCCT	GCCATAGAATAACCCTGGTCG
Set 43	GTCCTAGAAATGGTTCCACT	GCTCATGTAATTGAAACACCTG
Set 44	CTTCACCATCCTCCAAGC	AGATGAGGGCAATTAGCA
Set 45	ACCTTAGACGCTTCATGAT	AATGGTATTCCTGTGAGGG
Set 46	AGGGTTCTACTCAAAAGACC	TTTCATGTCATTGGTCGCAG
Set 47	CCTTCTAGGAGTCTGCCTA	CTTATTTAAGGGGAACGTATGG
Set 48	TCAACATAGCCGTCAAGG	TAGTCTTTCATCTTTCCCTTGCG
Set 49	ATTACAGCTAGAAACCCCG	GGTTGTTAAAGGGCGTATTGG
Set 50	TCTATGAGTTCCCCTACC	TTTTCGGCGGTAGAAGTA
Set 51	ATCACCTTAAGACCTCTGGT	GCCATAGAATAACCCTGGTCG
Set 52	GTCCTAGAAATGGTTCCACT	GTAGGGTCGAATCCGCAT
Set 53	TACTTCCACTACCATGAGCA	GACCATTTGAAGTCCTCG
Set 54	ACACTAATAGCCCTTCACAT	AATGGTATTCCTGTGAGGG
Set 55	AGGGTTCTACTCAAAAGACC	CTTCGATAATTCCTGAGATTGG
Set 56	AGAAGGAGCTACTCCCCA	TAGTCTTTCATCTTTCCCTTGCG

Forward and reverse primer sequences are listed for both the long PCR (Round 1 PCR) and short PCR (Round 2 PCR) reactions.

Table S2. Primer sequences used for PCR amplification of human single mitochondrion genomic samples. Related to Figure 1.

Long PCR Primers

	Forward	Reverse
Amplicon 1	GATCACAGGTCTATCACCCT	TAGCGTGGTAAGGGCGAT
Amplicon 2	ACAGACCAAGAGCCTTCAA	AAGGTTGGGGAACAGCTA
Amplicon 3	CAAGCCAACGCCACTTATCC	TGAAGTAGGAACCAGATGTCG

Short PCR primers

	Forward	Reverse
Set 1	GATCACAGGTCTATCACCCT	TGGCTAAGGTTGTCTGGT
Set 2	TAGCAAGGACTAACCCCT	CGTAGGGGCCTACAACGT
Set 3	TCTACATCACCGCCCCGA	TAGCGTGGTAAGGGCGAT
Set 4	ACAGACCAAGAGCCTTCAA	AATGAGCCTACAGATGATAGGA
Set 5	TCATGATTTGAGAAGCCTTCG	GTGTGCCTTGTGGTAAGAA
Set 6	CCTACTCATGCACCTAATTGG	AAGGTTGGGGAACAGCTA
Set 7	CAAGCCAACGCCACTTATCC	GCCGATGAACAGTTGGAA
Set 8	AGTTGATGATACGCCCGA	AGCGATGGCTATTGAGGA
Set 9	CCCCATAAATAGGAGAAGGCT	TGAAGTAGGAACCAGATGTCG
Set 10	GATCACAGGTCTATCACCCT	TCGATTACAGAACAGGCTCC
Set 11	CCCACGTAAAGACGTTAGGTC	ACTGGTGATGCTAGAGGTG
Set 12	ACCTGTATGAATGGCTCCA	TGAGACTAGTTCGGACTCC
Set 13	CCCTCACCACTACAATCTTCC	TAGCGTGGTAAGGGCGAT
Set 14	ACAGACCAAGAGCCTTCAA	ATGGTGTGCTCACACGAT
Set 15	CCTCCGCTACCATAATCATCG	CCTAATGTGGGGACAGCT
Set 16	CAGATGCAATTCCCGGAC	AGGCCTTTTTGGACAGGT
Set 17	CCCCTAGAAGTCCCACTC	AAGGTTGGGGAACAGCTA
Set 18	CAAGCCAACGCCACTTATCC	TCTTGTGAGCTTTCTCGGT
Set 19	AGGTCAACCTCGCTTCCCCAC	AAAGGCGGTTGAGGCGTCTG
Set 20	CGAGGCCTATATTACGGATCAT	TGAAGTAGGAACCAGATGTCG
Set 21	GATCACAGGTCTATCACCCT	CAGCTATCACCAGGCTCG
Set 22	AGAGGAACAGCTCTTTGGA	TTGGGCTACTGCTCGCAG
Set 23	CCTCTGATTACTCCTGCCA	TAGCGTGGTAAGGGCGAT
Set 24	ACAGACCAAGAGCCTTCAA	GCTGCATGTGCCATTAAGAT
Set 25	AAGTAGGTCTACAAGACGCT	CCCTGTTAGGGGTCATGG
Set 26	CCACTCCATAACGCTCCT	AAGGTTGGGGAACAGCTA
Set 27	CAAGCCAACGCCACTTATCC	TCTGCTCGGGCGTATCAT
Set 28	GCCCTTCTAAACGCTAATCCA	TGATTGTTAGCGGTGTGG
Set 29	TCATTATTCTCGCACGGACTA	TGAAGTAGGAACCAGATGTCG

Forward and reverse primer sequences are listed for both the long PCR (Round1 PCR) and short PCR (Round 2 PCR) reactions.

Supplemental Experimental Procedures:

Subjects. Mouse tissue was collected under University of Pennsylvania IACUC Protocol# 804867. Postnatal day 1 pups from which cultures were derived were not sexed for these studies. Pups were not sexed because cultures were derived from multiple pups provided by a shared resource. Human brain tissue was collected at the Hospital of the University of Pennsylvania under Institutional Review Board approval # 816223, using standard operating procedures for enrollment and consent of patients for patient numbers 50 (63 year old female) and 8 (65 year old female). See Supplemental Materials.

Cell Culture. Primary cortical neuronal, astrocyte or combination cultures were plated and maintained as described previously with some modifications (Kaech and Banker, 2006). Briefly, cortical neurons and/or astrocytes were isolated from postnatal day 1 mouse pups and dissociated with trypsin followed by pipetting prior to live cell counting with trypan blue and plating on poly-D-lysine/laminin coated 12-mm coverslips. Neuronal cultures were maintained in MEM with B27 supplement, astrocyte cultures in MEM with 10% FBS, co-cultures in MEM with B27 supplement and 1% FBS. Cultures of human cells were prepared and maintained as described previously (Spaethling et al., 2017). A 5 × 5 × 5 mm block of cortical tissue was resected as part of a neurosurgical procedure for the treatment of epilepsy or brain tumors. For transport (approximately 10 minutes from surgery to lab), this tissue was immediately transferred to a sterile tube containing ice-cold aCSF (2 mM $CaCl_2$-$2H_2O$, 10 mM glucose, 3 mM KCl, 26 mM $NaHCO_3$, 2.5 mM NaH_2PO_4, 1 mM $MgCl_2$-$6H_2O$, and 202 mM sucrose, perfused with 95%/5% O_2/CO_2). Upon receipt, tissue was digested with papain (20 U, Worthington Biochemical) and incubated for 10–15 min at 37°C. The reaction was stopped by addition of Leupeptin (a papain inhibitor, 100 μM, Sigma-Aldrich. Dissociated tissue was centrifuged (1,500 rpm for 3 min) and the supernatant subjected to gentle mechanical dissociation with a fire-polished glass Pasteur pipette. Live cells were stained with trypan blue and counted using an Autocounter (Invitrogen). Cells were plated on poly-L-lysine-coated 12-mm coverslips at a density of 3 × 10^4 cells/coverslip. Cultures were maintained at 37°C under 95% humidity and 5% CO_2 in medium (Neurobasal supplemented with B27 (1%) and penicillin/streptomycin (1%). The medium was changed by replacing 50% with fresh medium every 3 days.

Primer Design. All primers were designed by exhaustive computational analysis to minimize secondary structure and cross interactions and to maintain compatible melting temperatures within each set of PCR primers. The nested set of long PCR primers were designed by first generating three sets of primers that would create amplicons of approximately 5 Kb such that each set was offset from each other. We then designed short PCR primers again with overlapping offsets such that the set of amplicons exhaustively covered the long PCR amplicons (Fig. 1B).

Mitotracker Loading and imaging of cells. One coverslip of cells was transferred to one well of a 4 well dish containing 25 nM Mitotracker Red CMXRos (ThermoFisher Scientific, M-7512) in 500 μL 50% conditioned/50% fresh media. Mitotracker loading was performed at 37°C with 5% CO_2 for 20 minutes before transferring the coverslip to fresh 50% conditioned/50% fresh media and returning to the incubator for 10 minutes. The coverslip was then transferred to a low profile imaging chamber for imaging and sample collection. Samples were imaged with a Zeiss 710 LSM using the Alexa568 preset filter settings to image Mitotracker Red CMXRos and transmitted light for cell morphology. Samples were continuously perfused with normal saline (140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 16 mM glucose, 10 mM HEPES, pH 7.3) in between isolations.

Mitochondrial DNA Library construction and Sequencing. Library construction was performed using the Ilumina Truseq DNA nano library construction kit according to manufacturer's instructions. Successful short PCR reactions were pooled together and cleaned with a 0.5x AMPURE XP bead clean up. Based on concentrations of the pooled short PCR reactions obtained from running samples on an Agilent DNA 7500 chip on the Agilent Bioanalyzer, 247 ng of each pool was made up in a final volume of 130 μL of resuspension buffer, transferred to an AFA Fiber Pre-Slit Snap-Cap 6x16 mm microTUBE (Covaris 520045) and sheared on a Covaris S2 focused-ultrasonicator with the following settings to achieve an average insert size of 350 bp: Duty Cycle:10%, Intensity:5, Cycles/burst:200, Time: 45s). Sheared DNA was then quantified on an Agilent High Sensitivity DNA kit on the Agilent Bioanalyzer or the Agilent High Sensitivity D5000 kit on the Agilent Tapestation 2200 before inputting into the 350 bp insert workstream of the library kit protocol. Single mitochondrion libraries were pooled 24 samples per lane and run in rapid mode on a HiSeq 2500 with 2x101bp paired end reads.

Variant calling from RNASeq. The SNPiR pipeline (Piskol et al., 2013) was implemented as follows: The burrows-wheeler aligner (BWA) was used to map RNA-Seq reads derived from sequencing of mouse or human single cells using the commands "bwa aln fastqfile" and "bwa samse –n4". We only considered positions covered by the RNA-Seq for variant calling if the following criteria were met: (A) read depth coverage > 50, (B) baseQuality >

0, (C) mappingQuality > 0, (D) read frequency of minor allele ≥ 0.04%. SAMtools rmdup (Li et al., 2009) was used to remove identical reads derived from PCR duplicates that mapped to the same location.

Effect of variants on tRNA and protein structure. For position 3816, located in the gene encoding the mitochondrial tRNA for glutamine, the secondary structure was obtained from the mitotRNAdb maintained by the Universities of Leipzig and Strasbourg and the output structure used to indicate the location of the variable position in this tRNA (Jühling et al., 2009). For position 9027, located in the gene encoding mitochondrial cytochrome c oxidase subunit 3, the secondary and tertiary structure of this subunit was modeled by inputting UnitProt entry number P00416 (COX3_MOUSE) into the protein model portal for structural analysis (Schwede et al., 2009). The structure was inferred from the 85% sequence homology with bovine (*Bos Taurus*) heart cytochrome c oxidase protein in the fully oxidized state (1V54), whose structure was determined by x-ray crystallography at a resolution of 1.8 angstroms. At this level of structure resolution and sequence identity, the structural predications for the target (here the mouse protein) are typically correct. This particular subunit of the COX enzyme is highly conserved across Eukaryotes based on protein sequence alignments.

PCR error model. Here we use a model-based calculation to derive the probability of observing variant allele whose read frequency is greater than some threshold value, θ. Assume N cycles of PCR reactions resulting in 2^N total molecules. If a mutation occurs in the 1st PCR cycle, then 50% of the final molecules will have this mutation. In general, if a mutation happens in the *i*th cycle, $\frac{2^{N-i}}{2^N} = 2^{-i}$ proportion of the final molecules will have this mutation. Let *n* be the average sequencing depth, then we assume that out of 2^N total final molecules (including the library prep PCR cycles), we have sampled *n* total molecules. Let X be the number of sequenced reads carrying a non-reference mutation. We wish to compute $P(X > n\theta|n, i)$. Let *P(i)* denote the probability of a PCR mutation in the *i*th cycle. Then

$$P(X > n\theta|n) = \sum_{i=1}^{N} P(X > n\theta|n, i)P(i) \quad (1)$$

The probability *P(i)* of at least one mutation in the *i*th cycle is

$$P(i) = 1 - (1 - \varepsilon)^{2^{i-1}} \quad (2)$$

where ε is the PCR polymerase mutation rate. We note that (1) ignores the $O(\varepsilon^2)$ probability of convergent multiple mutations in the ith cycle. The term $\frac{2^i}{2} = 2^{i-1}$ denotes the number of lineages in the *i*th cycle where mutations may occur, where ½ is due to semi-conservative replication. Taq polymerase error rates are estimated to be between 1×10^{-4} to 1×10^{-5}. Therefore, using (2) for Taq,

P(i)\Cycle	1	2	3	4
Min	1×10^{-5}	2×10^{-5}	4×10^{-5}	8×10^{-5}
Max	1×10^{-4}	2×10^{-4}	4×10^{-4}	8×10^{-4}

The probability of sampling a molecule carrying a mutation from the *i*th cycle is 2^{-i} and we use the normal approximation to the binomial sampling process and assume the number *X* of sequenced molecules with variant mutation is

$$X \sim N(n \cdot 2^{-i}, \sqrt{n \cdot 2^{-i}(1 - 2^{-i})}).$$

In our experiments, our average sequencing depth was ~17,500; or, the number of molecules sampled was *n* = 17,500. Under these parameters $P(X > n\theta|n, i)$ is approximately 0 for values of *i* > 3. For values of $i \leq 3$, $P(X > n\theta|n, i)$is either approximately 1 or 0 for various values of θ. Table below shows the values of $P(X > n\theta|n, i)$ for $\theta = 0.1, 0.2, 0.3, 0.4$, and 0.5.

Cycle *i*\θ	0.1	0.2	0.3	0.4	0.5
1	~1	~1	~1	~1	0.5

2	~1	~1	~0	~0	~0
3	~1	~0	~0	~0	~0
4	~0	~0	~0	~0	~0

Combining the two tables in Equation (1), we obtain the following table for $P(X > n\theta | n = 17{,}500)$:

Table 1: Taq-related probability of observing false positive variant at frequency greater than θ.

$P(X > n\theta \| n, i)$ \ θ	0.1	0.2	0.3	0.4	0.5
Min	7×10^{-5}	3×10^{-5}	1×10^{-5}	1×10^{-5}	0.5×10^{-5}
Max	7×10^{-4}	3×10^{-4}	1×10^{-4}	1×10^{-4}	0.5×10^{-4}

For the enzyme Q5, the estimates of error rates are more than 100-fold lower. If Q5 enzyme is used in the 1st four PCR cycles, Table 1 can be simply scaled to

Table 2: Q5-related probability of observing false positive variant at frequency greater than θ.

$P(X > n\theta \| n, i)$ \ θ	0.1	0.2	0.3	0.4	0.5
Min	7×10^{-7}	3×10^{-7}	1×10^{-7}	1×10^{-7}	0.5×10^{-7}
Max	7×10^{-6}	3×10^{-6}	1×10^{-6}	1×10^{-6}	0.5×10^{-6}

Shared SNV sites for multiple mitochondria. Based on the PCR-derived false positive rate per site, the probability of observing k or more samples with a variant call at a particular site by misincorporation error is approximately $C(n,k)\times5.13\times10^{-4k}$, where n =118 is the number of samples in our mouse dataset. Using conservative Bonferroni correction over the ~16,000 sites of the mitochondrial genome, the probability, p, of observing k = 2, 3, 4 samples with a variant call are, p > 1, 0.5, 0.0085, respectively. For human samples with n =22, the Bonferroni corrected probability of k = 2, 3, 4 samples are p = 0.1896, 0.000126, and > 10^{-7}, respectively.