High-resolution imaging reveals compartmentalization of mitochondrial protein synthesis in cultured human cells

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Human mitochondria contain their own genome, mitochondrial DNA, that is expressed in the mitochondrial matrix. This genome encodes 13 vital polypeptides that are components of the multi-subunit complexes that couple oxidative phosphorylation (OXPHOS). The inner mitochondrial membrane that houses these complexes comprises the inner boundary membrane that runs parallel to the outer membrane, infoldings that form the cristae membranes, and the cristae junctions that separate the two. It is in these cristae membranes that the OXPHOS complexes have been shown to reside in various species. The majority of the OXPHOS subunits are nuclear-encoded and must therefore be imported from the cytosol through the outer membrane at contact sites with the inner boundary membrane. As the mitochondrially encoded components are also integral members of these complexes, where does protein synthesis occur? As transcription, mRNA processing, maturation, and at least part of the ribosome assembly process occur at the nucleoid and the spatially juxtaposed mitochondrial RNA granules, is protein synthesis also performed at the RNA granules close to these entities, or does it occur distal to these sites? We have adapted a click chemistry-based method coupled with stimulated emission depletion nanoscopy to address these questions. We report that, in human cells in culture, within the limits of resolution and electron microscopy, data were produced that were consistent with mitochondrial DNA (mtDNA)-encoded complex V subunits being synthesized predominantly on the CMs, while complex III and IV components were synthesized both at the IBM and CM. This approach is not feasible in human cells, as, with the exception of TACO1 (26), human mitochondria do not contain translational activators; moreover, the majority of mtDNA-encoded human proteins are components of complex I, an OXPHOS complex that is absent in S. cerevisiae. As translational activators were used as surrogate markers for translation, a direct assay for protein synthesis would now be possible.

Significance

In mitochondria from various species, the OXPHOS complexes reside mainly in the invaginated cristae membranes, as opposed to the inner boundary membrane (IBM) that parallels the mitochondrial outer membrane. However, the IBM contains dynamic contact sites enriched for translocases that import proteins from the cytosol. As the majority of OXPHOS components are imported and need to be integrated in assembly with the mtDNA-encoded components, where does intramitochondrial translation occur? Here we report: 1) a method for visualizing protein synthesis in human mitochondria at super resolution; 2) that synthesis is enriched at cristae membranes, in preference to the IBM; and 3) that sites of translation are spatially separated from RNA granules where RNA processing, maturation, and ribosome assembly occur.

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The authors declare no competing interest.

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ideal. One study has utilized a pulse-labeling click-chemistry method to directly measure mitochondrial protein synthesis in intact human cells (27). However, the limited resolution and depth of analysis meant many spatial characteristics of newly synthesized mitochondrial proteins remained undefined. In particular, no information on the submitochondrial localization of protein synthesis in human cells could be obtained.

We report here a comprehensive set of analyses that directly measure spatiotemporal kinetics of mitochondrial protein synthesis. Click chemistry (28) is the technique of choice for this approach, appealing to cell biologists as it utilizes free azide or alkyne labeling moieties that are rarely found in cells. The noncanonical methionine analogs homopropargylglycine (HPG) or azidothiophosphate (AHA) can be substituted for methionine and subsequently visualized in fixed cells with azido or alkyne fluorophores, respectively, in a process referred to as fluorescent noncanonical amino acid tagging, or FUNCT (29, 30). We have adapted initial protocols (27, 31) and used HPG while inhibiting cytosolic protein synthesis to specifically visualize mitochondrial protein synthesis with both confocal microscopy and super-resolution stimulated emission depletion (STED) nanoscopy. Signals reporting protein synthesis can be detected in various human cell lines after 5 min. Over 90 min of HPG labeling, the proportion of translationally active mitochondrial network (~50%) remained relatively unchanged. Using this method, we can report that the majority of protein synthesis is first detected at the CMS, colocalizing with mitoribosomal proteins and the translational couc-tivator TACO1. Further, our STED nanoscopy revealed that signal initiates mainly at sites separated from RNA granules, suggesting either that, if the mitoribosome is loaded with mt-tRNA close to the RNA granule, it must be able to travel to the CM prior to synthesis, or that the mitoribosome is loaded at the CM itself.

Results

Measuring Spatiotemporal Kinetics of Mitochondrial Protein Synthesis in Human Cells. Originally, a method had been described to measure newly synthesized cytosolic proteins in intact cells by immunofluorescence. This involved cells in methionine-free media being incubated with the alkyl or azido-methionine derivatives HPG or AHA, respectively. These methionine analogs were incorporated into growing polypeptides, with visualization occurring after fixation and copper-catalyzed cycloaddition of the noncanonical amino acid tagg-ing, to exclusively measure mitochondrial protein synthesis, and further refined previous techniques (SI Appendix, Figs. S1–S3) (27, 31). To ensure the method could be widely applied and that observations were robust, all experiments were performed with at least three types of human cultured cells unless otherwise indicated. The cell lines included two transformed lines (HeLa and U2OS) and primary dermal fibroblasts. As can be seen in Fig. 1A (U2OS cells) and SI Appendix, Fig. S2 (HeLa, fibroblasts), co-treatment of cells with cycloheximide (to inhibit cytosolic protein synthesis) and HPG allowed visualization of a signal that colocalized with the mitochondrial network. The HPG signal was lost upon simultaneous inhibition of cytosolic and mitochondrial protein synthesis (Fig. 1 A, Lower), demonstrating that the signal exclusively represented HPG-labeled mitochondrial synthesis. Although this demonstrated detectable mitochondrial translation, it did not determine whether HGylation of mt-tRNA^Met could be used to initiate synthesis. To address whether the signal represented only elongation of previously initiated proteins, cells were pretreated with puromycin to terminate synthesis prior to addition of HPG. As shown in SI Appendix, Fig. S3, mitochondrial derived signal was observed after puromycin pretreatment, consistent with de novo initiation of translation. This indicated that HGylated mt-tRNA^Met could both initiate synthesis as well as contribute to elongation. It is tempting to infer that a subset of HGylated mt-tRNA^Met could have been formulated by mitochondrial methionyl-tRNA for-myltransferase, mimicking the endogenous initiation mechanism (33), but recent data suggest that, under certain circumstances, initiation in mitochondria may occur without formulation of an initiating mt-tRNA^Met (34).

To determine whether the mitochondrial FUNCAT assay could be utilized to characterize the location and rates of mitochondrial translation, HPG incorporation in the mitochondrial network was measured over a time course (Fig. 1B). The rate in fibroblasts was relatively linear over a 45-min pulse, but a clear plateau was evident by 90 min (Fig. 1 B, Upper), which reflected trends seen in 35S-methionine (35S-met) radiolabeling of mitochondrial protein synthesis after similar inhibition of cytosolic protein synthesis.

It is not currently known whether translation occurs in discrete foci or is evenly distributed throughout the mitochondrial network. Analysis of fibroblasts revealed that, after a 15-min pulse, a reasonably homogenous pattern of synthesis was present across the network (Fig. 1 B, Lower). Longer pulses in these and HeLa cells reflected only an increase in pixel intensity [pseudocolored as a lookup table (LUT)] rather than redistribution of HPG signal (Fig. 1). More detailed analysis revealed protein synthesis to be modestly but significantly enriched in all three cell lines tested in perinuclear compared to peripheral mitochondria (SI Appendix, Fig. S4).

The established method for visualizing human mitochondrial protein synthesis requires 35S-met metabolic labeling (35) and has been adopted by many mitochondrial laboratories over the past 20 y. We attempted to visualize the newly synthesized HGylated proteins in gel-based systems to avoid the dependency on radiolabel. Following HPG pulse labeling, mitochondria were isolated and HGylated proteins labeled with picolyl biotin, permitting visualization after transfer. Robust incorporation of HPG into the majority of mitochondrial proteins can be seen (Fig. 1C), with a band pattern resembling previous reports for 35S-met gels. Labeling was eliminated by chloramphenicol treatment, confirming that the signal represented mitochondrially encoded proteins. However, despite trialing many combinations, our optimized protocol and gel systems were unable to separate and identify all of the 13 species (SI Appendix, Fig. S5). In the future, to fully optimize the procedure and resolve all 13 polypeptides, it may be necessary to use gradient gel systems, as recently reported (36), and, to unambiguously assign all species, it will be important to use patient cell lines that carry defined mutations in each of the polypeptides.

Turnover of Newly Synthesized HGylated Mitochondrial Proteins.

Although it is not immediately relevant to the question of where protein synthesis is occurring in the mitochondrion, we were interested to know whether polypeptides containing multiple HPG molecules were stable. A recent report suggested that, in HeLa cells, the majority of subunits are synthesized in substantial excess and the unassembled components are generally degraded within 3 h, while the fully assembled complexes are highly stable (37). We therefore elected to perform a pulse-chase experiment over 36 h (Fig. 2A). Following HPG treatment (2 h), both HPG and the reversible cytosolic protein synthesis inhibitor cycloheximide were removed and methionine added for the chase periods indicated. At 3 h post HPG pulse, ~87 ± 15% of the signal remained, suggesting that the majority of newly synthesized proteins were not rapidly degraded. Although there was a subsequent decrease after 6 h (36 ± 16%), inferring that a proportion of the newly synthesized mitochondrial protein is not assembled,
Mitochondrial FUNCAT Can Be Used to Identify Mitochondrial Protein Synthesis Defects. Traditionally, to determine whether individuals have defects in mitochondrial protein synthesis, patient-derived cell lines have been subjected to metabolic labeling with $^{35}$S-met in the presence of a cytosolic protein synthesis inhibitor (35). One of our goals was to establish mitochondrial FUNCAT as a tool that overcame the need for radiolabel in such assays. To establish whether our methodology could be applied to identify such defects, we used our assay to measure mitochondrial protein synthesis normalized against mitochondrial unit area in control fibroblast and two patient cell lines. Both patients carried defects in mitochondrial protein synthesis: a homozygous exonic deletion [c.210delA, p.(Gly72Alafs*13)] frameshift mutation in the release factor C12orf65 (38) and a mutation in the mitoribosomal subunit mS22. In both cases, a substantially lower signal was noted after 45 min when compared to controls (Fig. 2 B and C), but there was no such marked variation in the proportion of translationally active network (Fig. 2D). Importantly, by 90 min, the difference in signal between patient and control lines was abolished (SI Appendix, Fig. S6). This emphasized that kinetics have to be carefully established for each cell line. These data confirm that the mitochondrial FUNCAT assay is suitable for identifying and measuring mitochondrial protein synthesis defects and potentially any changes in distribution of signal within the network.

Newly Synthesized Protein Is Detected Predominantly at the CMs. The main focus of this study was to establish the submitochondrial localization of protein synthesis. To achieve this goal, we adapted the mitochondrial FUNCAT technique to enable visualization by super-resolution STED nanoscopy. One strength of FUNCAT is that fluorophores are attached directly to the methionine retention of signal at 36 h (16 ± 4%) was consistent with incorporation of at least a subset of labeled protein into stable complexes.

**Fig. 1.** Translation of mtDNA-encoded proteins can be monitored by incorporation of HPG in a time-dependent manner, revealing the distribution across the mitochondrial network. (A) U2OS cells were pulsed with HPG (25 min) and cycloheximide in the absence (Upper) or presence (Lower) of chloramphenicol (CAP). Cells were then fixed, underwent the click reaction, and were stained with antibodies against ATP5I. (B) Control fibroblasts were cultured in HPG with inhibition of cytosolic translation by cycloheximide for the time periods indicated. Postfixation, click reactions were performed and samples immunostained as for A. All images are representative. (Scale bars: 10 μm.) Relative pixel intensities of HPG are presented as pseudocolor-coded LUTs. The total HPG incorporation as a marker of mitochondria–protein synthesis was plotted over a 90-min time course (Upper). The proportion of the mitochondrial network stained with HPG reflecting how much of the reticulum is translationally active was calculated as a percentage at each time point from multiple cells from three independent repeats (n = 4, 7.5 min; n = 12, 15, 25, 45, 90 min) and is presented graphically (Lower). (C) Wild-type HEK293 cells were incubated in the presence or absence of inhibitors [emetine and chloramphenicol (CAP)] followed by addition of HPG. Mitochondria were isolated and the click reaction performed as described in Materials and Methods. Samples were separated by 12% Bis-Tris PAGE and biotinylated proteins visualized with Streptavidin-HRP and ECL. Asterisks indicate endogenously biotinylated proteins.
analog by the copper-catalyzed alkyne–azide reaction. We were able to optimize the technique using the picolyl Alexa Fluor 594 azide. Colocalization was then determined with various markers using immunofluorescence techniques as described. We first assessed whether newly synthesized proteins could be identified away from the mitochondrial outer membrane (Fig. 3A). HPG was pulsed for 30 min, and the signal was compared to that produced from antibodies specific for the outer membrane marker TOM20. High-resolution imaging indicated that newly synthesized proteins are located internal to the outer membrane (Fig. 3A and B). Further, the HPG signal clearly shared a location with ATP5I, a component of the FoF1 ATP synthase and member of the CM (Fig. 3B and C). Therefore, to assess more precisely where in the IMM the highly hydrophobic mtDNA-encoded proteins were being inserted, we quantified colocalization with markers from the IBM (TIM23, a component of the inner membrane translocase) and CJs (MIC60, a member of the MICOS complex) and a second marker of the CM (COXI, a component of cytchrome c oxidase or complex IV). Images in Fig. 3D confirm that the majority of HPG signal is found in the CM, colocalizing with COXI, where the IBM begins to invaginate to form the distinct compartment of the CM (Fig. 3D and SI Appendix, Fig. S7, ATP5I cf. MIC60). Again, the majority of the HPG signal is distinct from and internal to MIC60 puncta, consistent with the newly synthesized proteins being inserted into the CMs. Quantification using Manders’ colocalization coefficient ($M_2$) indicated that, of the HPG signal, $50.8\pm8.8\%$ ($M_2=0.508$) associated with the cristae (COXI), while only $21.5\pm8.9\%$ colocalized with the IBM marker TIM23 and $12.6\pm5.4\%$ with CJ marker MIC60. Analysis with Spearman’s rank correlation coefficient also revealed an approximately threefold increase in correlative relationship with COXI (Fig. 3D).
between HPG and COX1 or HPG and ATP5I signals compared to markers for the other submembrane compartments (SI Appendix, Fig. S12), a phenomenon also observed over longer pulses. Similar labeling is also seen when comparing to MIC10, a second member of the MICOS complex and marker of the CJ (SI Appendix, Fig. S7). To account for the possibility of movement of newly synthesized proteins within the 30-min pulse, a shorter (7.5-min) pulse was applied that revealed a similar ATPS (complex V beta subunit) distribution (SI Appendix, Fig. S8). However, the majority of the data analyzed were after a 15- or 30-min pulse, when the capacity to attain sufficient signal and signalto-noise were significantly improved. Taken together, our data support the majority of protein synthesis occurring at CMs.

**HPGylation Is Detected at Sites Proximal to Mitoribosomal Components as Well as Cristae.** Previous data showed that the majority of HPG signal could first be detected at the CMs. If this signal was a true marker of nascent protein synthesis, colocalization would be expected with markers of components of the mitoribosome, assuming that most of the components were in fully assembled mitoribosomes that were synthesizing protein. This would be consistent with a previous study using immunoelectron microscopy that reported an enrichment of a single yeast mitoribosomal subunit, YmL36, at the CMs (9), an observation consistent with our STED studies. Therefore, to assess the levels of colocalization between HPG signal and the mitoribosomes, we determined the position of three
small subunit proteins, \textit{uS15m}, \textit{uS17m}, and \textit{mS27}, and \textit{mL45} from the large subunit. As shown and quantified in Fig. 4 and \textit{SI Appendix, Fig. S9}, a 30-min pulse of HPG signal in U2OS cells colocalized strongly with all MRPs measured, and, notably, with the CM marker ATP5I (\textit{SI Appendix, Fig. S9 G and H}). A much more limited colocalization was seen between HPG and markers of other mitochondrial membrane compartments (\textit{SI Appendix, Fig. S9 G and H}). Further, a significant increase in colocalization of each MRP was observed with the CM (ATPS) in comparison to the CJ (MIC60) using immunofluorescence alone (\textit{SI Appendix, Fig. S10}).

TACO1 is thought to be a human equivalent of a yeast mitochondrial translational coactivator (26). We therefore reasoned that the intramitochondrial location of TACO1 could be a surrogate marker for nascent synthesis. U2OS lines were generated that inducibly expressed a C-terminal FLAG-tag version of human TACO1. Quantification of Manders’ coefficient revealed that, on induction, 74 ± 8.0% of the TACO1 signal colocalized with HPGylated protein after a 30-min pulse (\textit{SI Appendix, Fig. S11}). A similar correlation was noted with the CM marker ATP5I (63 ± 5.6%), but not with markers of other subcompartments (TOM20, 21 ± 8.5%; MIC60, 19 ± 7.2%). Taken together, these data are consistent with the majority of newly synthesized human mitochondrial protein being inserted into the CMs.

**Mitochondrial Protein Synthesis Colocalizes More Closely With Cristae Than With Mitochondrial RNA Granules.** Mitochondrial gene expression appears highly compartmentalized, with large, juxtaposed...
complexes (the nucleoid and the RNA granule) in the mitochondrial matrix as highlighted in the Introduction. Is it possible that translation also occurs within the mitochondrial RNA granule, or even in a third juxtaposed structure creating a form of production line to synthesize new proteins? In yeast mitochondria, this idea has been supported by coimmunoprecipitation, proteomics, and super-resolution microscopy studies. Those data revealed an association between the mitoribosomes and other gene expression proteins, leading to the concept of MIOREX, or mitochondrial organization of gene expression (40). To assess whether human mitochondria show a similar arrangement of associated complexes, we used STED nanoscopy to visualize mitochondrial protein synthesis (HPG) and antibodies to highlight two separate markers of the mitochondrial RNA granule (GRSF1, Fig. 5; and GRSF1 and FASTKD2, SI Appendix, Fig. S8). These figures highlight that the majority of HPG signal is spatially distinct from mitochondrial RNA granules. Quantification (Manders’ colocalization analysis) of data from the 15-min pulse-labeled U20S cells revealed that 64.4 ± 8.2% of the HPG signal colocalized with the mitoribosomal subunit uS15m but only 9.5 ± 2.9% with the RNA granule (GRSF1). The HPG signal was also enriched with the cristae marker ATP5l (62.7 ± 2.5%), with a ~10-fold enrichment compared to GRSF1 (54.7 ± 4.7% vs. 5.4 ± 3.0%) at 30 min (SI Appendix, Fig. S12). Even as early as a 7.5-min pulse, the staining profile of HPG more closely resembled ATP5l than FASTKD2 or GRSF1 (SI Appendix, Fig. S8, compare white overlap in merge panels of A and B vs. C). Indeed, a similarly low association was also noted between GRSF1 and the translational activator TACO1 (SI Appendix, Fig. S11). Taken together, our data show little evidence for compartmentalization of protein synthesis within RNA granules.

Discussion

We report that mitochondrial FUNCAT is a robust method for visualizing protein synthesis in human cells. This technique represents a significant development in methodology to investigate human mitochondrial protein synthesis, in particular with an adaptation to higher-resolution confocal and STED microscopy. Unlike 35S-met radiolabeling that was limited to scintillation counting or gel-based display formats, this approach can visualize spatial information on the distribution of translation within the mitochondrial network. When implemented with super-resolution microscopy and combined with immunofluorescence, as we have here, it allows us to gain a better understanding of how and when different machineries involved in all aspects of gene expression are integrated and how this is affected by different disease conditions. Currently, it is not possible for us to confidently produce consistent data with HPG pulse times shorter than 10 min in all cell types. For this reason, we have exercised caution in our interpretations and conclusions. We believe our data are consistent with the majority of protein synthesis occurring at the CM and distal from the nucleoid or RNA granule. One alternative

FIG. 5. Spatial distribution of mitochondrial RNA granules and intraorganellar translation. U2OS cells were pulsed with HPG (15 min) prior to fixation and click reactions as described in Materials and Methods. Immunostaining was performed with antibodies against markers of the mitochondrial RNA granules (GRSF1, A), cristae (COXI, B; ATP5l, C), or IBM (TIM23, D). Relative HPG pixel intensities represented as pseudocolor-coded LUTs as well as surface-rendered (SR) representations of the merged data. Deconvolved STED microscopy images are representative of three independent repeats. (Scale bars: 1 μm.)
Materials and Methods

Unpublished work was based on quantifying the nanoparticle density of mitochondrial protein synthesis within whole-cell confocal images, using Huygens software (Scientific Volume Imaging).

Confocal Imaging and Three-Dimensional STED Nanoscopy. Confocal imaging and three-dimensional (3D) STED nanoscopy were performed on a Leica TCS SP8 gSTED 3X microscope (Leica Microsystems) equipped with white light lasers, HC PL APO 100×/1.40 OIL STED WHITE objective, and 63×/1.40 Oil HC PL APO CS2 objective for confocal imaging. A voxel size of (35 to 40) × (35 to 40) × 130 nm (xyz) for z>63 confocal and (10 to 20) × (10 to 20) × 100 nm (xyz) for STED images was used. The fluorophore Alexa Fluor 532 was excited at 527 nm, and STED was performed at 660 nm for super-resolution imaging. Alexa Fluor 555 was excited at 555 nm, and STED was performed at 660 nm. Alexa Fluor 594 was excited at 590 nm and ATTO647N at 646 nm, while STED was performed at 710 nm for both fluorophores. Images were deconvolved and, where indicated, rendered into computer-generated 3D surface maps, using Huygens software (Scientific Volume Imaging).

Image Analysis. In each case, unless otherwise stated, 5 to 12 images were analyzed from a single representative experiment that was repeated at least three times with similar results. For determining amount and distribution of mitochondrial protein synthesis within whole-cell confocal images, using Huygens software (Scientific Volume Imaging).

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High-resolution imaging reveals compartmentalization of mitochondrial protein synthesis in cultured human cells
Bioorthogonal Noncanonical Amino Acid Tagging: Cell Culture and Click-Chemistry Labeling of Mitochondrial Translation in Human Cells

Bioorthogonal noncanonical amino acid tagging (NCATT) is a powerful technique that allows for the label-free visualization and functional analysis of mitochondria in living cells. This method involves the use of noncanonical amino acids (ncAAs) that are incorporated into proteins during translation, followed by the application of bioorthogonal reagents to visualize the tagged proteins. The process typically involves the following steps:

1. **Protein Synthesis with ncAAs**: Cells are cultured in media containing noncanonical amino acids (ncAAs), which are incorporated into proteins during translation. The incorporation of these ncAAs is mediated by the use of specific translation elongation factors that recognize the ncAAs.

2. **Bioorthogonal Reagents**: After protein synthesis, the cells are treated with bioorthogonal reagents that selectively label the ncAAs. These reagents are designed to be non-toxic and to selectively react with the tagged proteins, allowing for their visualization using fluorescence microscopy.

3. **Image Acquisition**: The tagged proteins are then imaged using confocal or super-resolution microscopy techniques to visualize the localization and dynamics of the tagged proteins within the mitochondria.

4. **Data Analysis**: The images are analyzed to assess the distribution and dynamics of the tagged proteins, providing insights into mitochondrial function and dynamics.

This technique has been applied to study various aspects of mitochondrial biology, including mitochondrial translation, dynamics, and function in both healthy and disease states. The ability to label proteins in a cell-selective manner makes NCATT a valuable tool for understanding the complex role of mitochondria in cellular processes.