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INVITED COMMENTARY

Multilevel heterogeneity of mitochondrial respiratory chain deficiency†

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†Invited commentary for Simard *et al.* A novel histochemistry assay to assess and quantify focal cytochrome c oxidase deficiency. *J Pathol* 2018; **245:** 311–323.

Abstract

Mitochondrial diseases are heterogeneous multisystem disorders that show a mosaic pattern of mitochondrial respiratory chain dysfunction. The mitochondrial DNA (mtDNA) mutation load is heterogeneous at multiple levels: across organs, between cells, and between subcellular compartments. Such heterogeneity poses a diagnostic challenge, but also provides a scientific opportunity to explore the biological mechanisms underlying the onset and progression of these disorders. A recent article in *The Journal of Pathology* described a novel histochemical technique – nitro blue tetrazolium exclusion assay (NBTx) – to quantify mitochondrial cytochrome *c* oxidase (COX, or complex IV) deficiency. This technique is rapid, cost-effective, and quantitative, and is more sensitive than previous histochemical methods. It can also be applied across model organisms and human tissues. The NBTx method should therefore be a useful diagnostic tool, and may catalyze research examining the cellular and subcellular mechanisms that drive the onset and progression of inherited and acquired mtDNA disorders.
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Mitochondrial DNA (mtDNA) encodes two rRNAs, 22 tRNAs, and 13 subunits of the respiratory chain, making it essential for normal mitochondrial function. Mutations of mtDNA can occur sporadically or be inherited, and result in multisystem disorders with wide-ranging clinical heterogeneity [1]. In part, this heterogeneity is due to the segregation of mtDNA mutations to different tissues and cells [2], and selective pressures that may impact the clonal expansion of mtDNA mutations throughout life. Such heterogeneity exists not only between organs and tissues of a given individual (for example, the brain may contain high mutation levels, but blood leukocytes may not [3]), but also between cells of the same organ, whereby two adjacent cells contain different levels of mutant mtDNA (Figure 1A,B). Furthermore, heterogeneity also exists at a deeper level of biological organization, across subcellular compartments of single cells. This is exemplified in neurons, in which single mitochondria may contain mtDNA mutations [4], and in human muscle fibers, in which spatially segregated subpopulations of mitochondria with mutant and normal genomes coexist within the cytoplasm (Figure 1A,C,D).

Although substantial advances have been made in the diagnosis and understanding of mitochondrial disease, this complex set of pathologies remain difficult to identify, diagnose, and treat. Even in the current genomic era, histological assessment of muscle biopsies is often the first step and a key element in the diagnostic algorithm to identify mitochondrial respiratory chain deficiency.

Moving beyond the homogenate

Enzymatic deficiency can be quantified from homogenates of skeletal muscle biopsies and dermal fibroblasts isolated and cultured from the skin [5], or with other polarographic methods used to measure mitochondrial respiration. Molecular analyses

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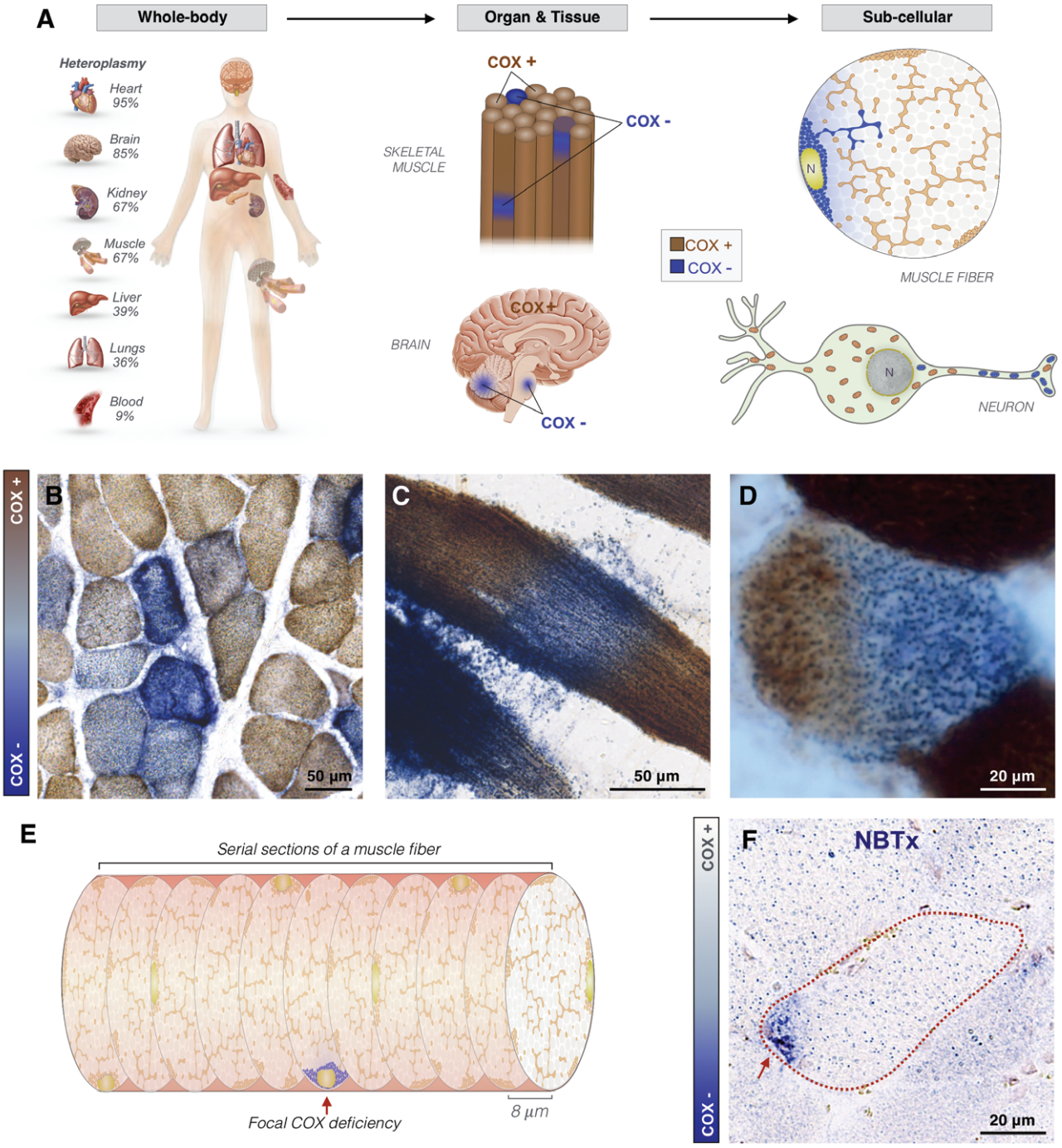

Figure 1. Multilevel respiratory chain heterogeneity mapped with histochemistry. (A) Schematic illustrating the heterogeneity of mtDNA mutation load and mitochondrial respiratory chain deficiency at the whole body level (left), at the tissue/organ level (center), and at the subcellular level (right). At the whole body level, mutation load (percentage heteroplasmy) varies between different organs, with most energetically demanding organs possibly having higher mutation loads [17]. At the organ level, there is regional enrichment of respiratory chain deficiency, with some completely COX-deficient (COX^{-}, blue), some intermediate (COX^{inter}) and some COX-positive (COX^{+}) cells. Similarly, single cells can show subcellular heterogeneity marked by an uneven distribution of mutant COX-deficient (blue) and normal (brown) mitochondria in different cytoplasmic compartments. Heteroplasmy levels shown for different organs are derived from three patients with the m.3243A > G $tRNA^{Leu(UUR)}$ mutation, adapted from Maeda *et al* [3]. N, nucleus. (B) Example of COX/SDH histochemistry in human skeletal muscle in cross-section, showing the heterogeneity of mitochondrial COX deficiency across different cells in the same biopsy. (C) COX/SDH histochemistry demonstrating heterogeneity of mitochondrial COX deficiency in a single human skeletal muscle fiber in longitudinal orientation. (D) COX/SDH histochemistry showing a partially COX-deficient and COX-positive human diaphragm muscle fiber in cross-section, presumably representing a transitory intermediate stage as mutant mitochondria clonally expand and propagate intracellularly from right to left. (E) Schematic of serial sections of a muscle fiber, with a focal region of COX deficiency spatially restricted to a single section. This illustrates the need for histochemical methods compatible with downstream molecular genetic analysis on the same section, such as the NBTx [13]. (F) Human skeletal muscle fiber in cross-section subjected to the NBTx demonstrating a small subsarcolemmal focal area of COX deficiency, presumably representing the origin and early stage of clonal expansion for *de novo* mtDNA defects that lead to widespread respiratory chain deficiency [15].

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such as western blotting and blue native polyacrylamide gel electrophoresis of tissues can also establish the abundance and assembly of respiratory chain (super)complexes. However, these methods rely on analyses of homogenates, which, given the heterogeneity of mtDNA defects, may obscure the clinically meaningful heterogeneity in mitochondrial disease.

Historically, in part to address this limitation, skeletal muscle biopsies of patients with suspected mitochondrial disease have been assessed with an array of histological techniques that allow resolution of individual cells. Two respiratory chain enzymes have been particularly studied: complex IV [cytochrome *c* oxidase (COX)] and complex II [succinate dehydrogenase (SDH)]. Because COX is partially encoded by mtDNA but SDH is entirely nuclear-encoded, mtDNA mutations selectively impair COX activity but leave SDH activity unaffected (or sometimes increased). This discriminant feature has been exploited in skeletal muscle cryosections with the use of sequential COX/SDH histochemistry *in situ* [6]. In cells with only normal mtDNA exposed to the histochemical buffer, both COX and SDH activities are normal, and the enzymes precipitate 3,3′-diaminobenzidine (DAB) (brown) and nitro blue tetrazolium (NBT) (blue), resulting in a dark brown appearance. However, cells with mtDNA defects selectively lose the COX-related brown precipitate, and appear blue. Thus, the presence of blue cells enables the clinician or researcher to identify COX deficiency and the likely presence of mtDNA mutations.

Although COX/SDH histochemistry is a rapid (~2 h) and cost-effective assay with which to assess COX activity, it is limited by at least three main pitfalls. First, the interpretation of COX/SDH histochemistry is mostly subjective, especially when cells show an intermediate loss of COX deficiency, and have a difficult-to-interpret appearance consisting of shades of brown, blue, and gray. Second, only cells with severe COX deficiency appear blue, which does not enable the investigator to detect subtle changes in COX activity or low levels of mtDNA mutation. Third, the chemical used to reveal COX activity, DAB, inhibits some downstream molecular analyses, such as real-time quantitative polymerase chain reaction (qPCR), that are used to quantify mtDNA mutation levels in microdissected cells [7,8].

Alternatives to COX/SDH

More recently, an immunofluorescence method was developed for quantitative analysis of complex I and complex IV protein levels at single-cell resolution [9]. This method is semi-automated, objective, and quantitative. It can detect subtle changes in proteins and thus overcomes the limited sensitivity of COX/SDH histochemistry. Furthermore, because it relies on antibodies rather than on the benzene derivative DAB to assess deficiency, it does not interfere with downstream qPCR analysis [10]. If used properly, this assay provides quantitative information on the abundance of specific subunit proteins of complexes I and IV, and outer membrane-bound markers of mitochondrial mass. However, it measures the presence of protein rather than enzymatic activity, and so may not always be as meaningful as COX/SDH histochemistry. In addition, although quantitative immunofluorescence is now being implemented in some diagnostic laboratories, in comparison with COX/SDH histochemistry, this approach is costly, time-consuming, and requires specialized expertise. Furthermore, in contrast to COX/SDH histochemistry, in which reagents are used ubiquitously (with variation in incubation times) on various tissue types and across different species, quantitative immunofluorescence approaches require substantial optimization and standardization, particularly in the choice of specific antibodies that may have immunoreactivity only in some tissues or species [11,12].

The elegant NBT exclusion assay (NBTx) assay recently described by Simard *et al* [13] provides an alternative to COX/SDH histochemistry with several advantages. The assay is cost-effective and more rapidly performed than COX/SDH histochemistry, but can detect subtle changes in COX activity. Because it does not rely on DAB, it is also compatible with qPCR. This is advantageous to investigators wishing to perform laser capture microdissection on a histochemically stained tissue section combined with downstream genetic analyses of the same section. A popular alternative approach consists of generating serial cryosections, the first of which is used for COX/SDH, and the second of which is used for SDH only (without DAB). The COX/SDH section is used to identify COX-deficient cells, which are subsequently matched onto the SDH-treated section for microdissection and genetic analyses. However, this process is laborious and certain tissues such as the brain contain small and highly complex (sub)cellular compartments that may not be available across serial sections (e.g. [14]). Combined histological and molecular analyses on such tissues is certainly favorable.

Furthermore, perhaps one of the most significant advances provided by the NBTx method over COX/SDH histochemistry may be its increased sensitivity and quantitative nature. Simard *et al* [13] demonstrate that cardiac muscle fibers that are seemingly COX-normal on the basis of COX/SDH histochemistry do, in fact, show COX deficiency when evaluated with the NBTx, and also provide a quantitative densitometric analysis to estimate the severity of the enzymatic defect. Because the NBTx is rapid, cost-effective, sensitive, and quantitative, and stains thousands of cells at once in a single sample, it may provide an unprecedented opportunity to screen and establish biochemical deficiency in a cell-specific manner in clinically relevant tissue samples and model systems.

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The need for subcellular resolution

Combined with microscopy, this assay can also detect small areas of COX deficiency – at the subcellular level. Postmitotic cells tend to be highly polarized, with subcellular compartments performing specialized functions. For example, neurons have somatic, dendritic and axonal compartments, each with distinct mitochondrial populations; and skeletal muscle fibers contain subsarcolemmal and intermyofibrillar mitochondria that differ in their morphology and functions. Microscopic methods allow investigators to biochemically map COX deficiency among mitochondrial subpopulations within single cells. This, in turn, will enable researchers to ask new kinds of questions about the mechanisms that regulate the intracellular distribution of mtDNA mutations, and possibly about the cytoplasmic origin of *de novo* mtDNA mutations, which are known to propagate and clonally expand within cells.

A recent study using a combination of COX/SDH histochemistry and immunofluorescence identified subcellular focal regions ('foci') of COX deficiency in human skeletal muscle fibers [15]. This highlights a new degree of tissue heterogeneity in skeletal muscle fibers (Figure 1D). Intracellular COX-deficient foci can be surprisingly small – presumably representing the early stage of clonal expansion, with few mutant mtDNA molecules. These respiratory chain-deficient foci occasionally span no more than ~10 µm in any direction. Therefore, given that cryosections are typically 5–10 µm, foci may only exist in a single section (Figure 1E). As demonstrated in Figure 1F, the NBTx will now enable clinicians and researchers to detect and analyze the genetic nature of subcellular foci of COX deficiency without compromising PCR chemistry. We also expect this method to yield more accurate and sensitive identification and quantification of COX-deficient foci than is possible with the COX/SDH reaction. Gaining access to subcellular information on the distribution and severity of mitochondrial respiratory chain deficiency should help us to understand the progression of genetic mitochondrial disorders.

Conclusion

Although there are numerous homogenate-based methods with which to assess the presence and activity of mitochondrial respiratory chain activity and protein abundance, many are confounded by the tissue-level and intracellular heterogeneity of mtDNA defects. Microscopy-based histochemical and immunofluorescence techniques that maintain cellular integrity are essential to: (1) adequately assess cellular aspects of disease status clinically; and (2) understand the cellular mechanisms underlying disease onset and progression. Advances in histological methods have established the association between mitochondrial genetics and cellular phenotypes such as biochemical deficiency patterns [10] and apoptosis [16], and have suggested new principles whereby respiratory chain deficiency originates and spreads intracellularly [15]. The new NBTx method by Simard *et al* [13] expands the current histological toolkit to investigate these and other clinically meaningful questions in mitochondrial biology.

Ethics statement

Ethical approval for the use of tissues was granted by the Newcastle and North Tyneside Local Research Ethics Committee (reference 16NE/0267) and McGill University Health Centre (BMB 06–018).

Author contributions statement

AEV and MP wrote the commentary and generated figures. AEV and MP performed histology.

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