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# From Transcriptomics to Mitochondrial Energetics

*Why Gene-Expression Panels Are a Confounded Proxy — and the Seahorse Assay a Direct Measure of Mitochondrial Function*

Transcriptomics, for all its genome-wide reach, is an indirect and heavily confounded proxy for mitochondrial function — while the extracellular-flux ("Seahorse") assay directly measures the bioenergetic phenotype the transcriptome can only gesture at.

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# Why Gene-Expression Panels Are a Confounded Proxy

Why differential-expression panels — genome-wide but indirect — are a confounded proxy for mitochondrial function, and why the extracellular-flux ("Seahorse") assay is a direct, functional measurement of the bioenergetic phenotype: oxygen-consumption rate, ATP-linked respiration, and spare respiratory capacity read as measured reserve.

ABSTRACT

# Abstract

Gene-expression profiling has become a default lens for interrogating chronic illness, including in the biotoxin field, where differential-transcription panels are used to infer inflammatory, metabolic, and mitochondrial "programs" and to stage disease. This monograph argues that transcriptomics, for all its genome-wide reach, is an indirect and heavily confounded proxy for mitochondrial function, and that the extracellular-flux ("Seahorse") assay is a direct, functional measurement of the bioenergetic phenotype the transcriptome can only gesture at. The argument proceeds in three moves. First, the central-dogma gap: steady-state mRNA abundance explains only about forty percent of the variance in protein level and still less of enzyme activity and pathway flux, and the two decouple further precisely during acute stress and proteome remodeling. Second, the confounder stack: whole-blood transcriptomics is a weighted average over a shifting mixture of cell types, is strongly time-of-day dependent, is perturbed by the blood draw and its processing, and is remodeled by medications, diet, age, and analytic choices — so a differential-expression "signal" may index none of the biology it is taken to index. Third, and most decisive within this framework: the mitochondrial integrated stress response (ISRmt), the very state at issue, is a translational reprogramming that suppresses global protein synthesis while selectively translating ATF4 and CHOP together with their circulating mitokine outputs FGF21 and GDF15 — so in the target state, transcript abundance and functional output are not merely loosely coupled but actively inverted. The Seahorse Mito Stress Test, by contrast, measures the oxygen-consumption rate of living cells and resolves it — through oligomycin, FCCP, and rotenone/antimycin A — into basal respiration, ATP-linked respiration, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration. These are the integrated functional output of every upstream layer of regulation, and spare respiratory capacity is a direct, quantitative rendering of the reserve this framework treats as governing. The two methods are complementary — transcriptomics for genome-wide discovery, flux analysis for functional truth — but for the specific question "is mitochondrial function impaired, and how much reserve remains," the Seahorse assay measures what transcriptomics can only infer.

SECTION ONE

# The Appeal and the Problem: Reading Function Off Transcripts

The appeal of transcriptomics is real and worth stating plainly. A single assay returns a genome-wide, hypothesis-free census of which genes are being transcribed, from a small sample, at industrial scale. A heat map of up- and down-regulated genes looks like a direct readout of what a cell — or a patient — is doing. In the biotoxin and chronic-illness field, differential-expression panels are used in exactly this spirit: to name inflammatory, metabolic, mitochondrial, and coagulation "programs," to sort patients, and to track response. The genome-wide reach is genuinely powerful, and nothing here disputes its value for discovery. The problem is what happens when a transcriptome is asked to serve as a functional readout — when transcript movement is

taken to report the state of a pathway. A transcriptome is, mechanically, a list of mRNA copy numbers. Copy number is several regulated steps removed from function, and reading function off it quietly assumes three things: that the transcript predicts the protein, that the protein predicts the flux, and that the transcript moved because of the biology of interest rather than because of a confounder. Each assumption is weak on its own. Stacked, they are fragile. And in the mitochondrial case specifically, the third assumption is frequently false and the first can point in the wrong direction. This monograph makes that case in three steps — the central-dogma gap between transcript and function (Section Two), the confounders that can manufacture a "signature" with no change in the underlying biology (Section Three), and the mitochondrial integrated stress response, in which the disease state itself inverts the transcript-to-function relationship (Section Four) — and then sets against it the extracellularflux assay as a direct functional measurement (Sections Five and Six), before returning to a fair division of labor between the two (Section Seven).

SECTION TWO

# The Central-Dogma Gap: mRNA Is Not Protein, and Protein Is Not Flux

Between a transcript and a functional consequence lie several independently regulated layers, each of which loosens the link. mRNA does not predict protein. Across large paired datasets in human and model systems, steady-state mRNA abundance explains on the order of forty percent of the variance in protein abundance ($R^2 \approx 0.4$); most of the variation in protein level is set after transcription. mRNA half-lives are short — on the order of a few hours — while proteins persist far longer, so a transcript is a fast, noisy leading indicator of a protein level, not a measure of it. Splicing, mRNA export and decay, microRNA regulation, and above all translation efficiency intervene between the transcript and the protein. Protein does not predict activity. Even where protein amount is known, functional output depends on post-translational modification, allosteric regulation, cofactor and substrate availability, and assembly into higher-order complexes. Oxidative phosphorylation is built from multi-subunit complexes and supercomplexes whose function depends on correct stoichiometry and assembly, not on the abundance of any one subunit's mRNA.

Activity does not predict flux. Pathway flux is governed by demand, substrate supply, and thermodynamics — membrane potential, the ADP/ATP ratio, oxygen — as much as by enzyme abundance. A cell can hold ample enzyme and still run little flux, or the reverse. Mitochondria double the problem. The respiratory chain is encoded by two genomes: thirteen core subunits are transcribed from mtDNA, the rest from the nucleus. Mito-nuclear stoichiometry, mtDNA copy number, and heteroplasmy shape function and are largely invisible to standard nuclear RNA-seq. And there is a direction trap unique to energetics: an energy-starved cell often up-regulates OXPHOS and biogenesis transcripts precisely because function is failing — a compensatory signal in which the transcript rises while capacity falls. A clean, replicated transcript change can therefore point the wrong way about the very thing it is taken to measure. Key point. mRNA is a leading, noisy indicator of one step in a multi-step chain to function — roughly forty percent of protein variance at that first step alone — and each subsequent step (protein, activity, flux) loosens the link further. Reading bioenergetic capacity off transcripts compounds several weak links, and under compensation it can invert.

SECTION THREE

# The Confounders of Blood Transcriptomics

Beyond the dogma gap, blood-based transcriptomics carries a stack of confounders that are not random noise to be averaged away. Several are structural: they can generate an entire differential-expression "signature" with no change in any cell's actual biology. Cell-type composition — the dominant confounder. A whole-blood or PBMC transcriptome is a weighted average over a mixture of cell types whose proportions shift with health and state. An apparent change in a gene may reflect a shift in the neutrophil-to-lymphocyte ratio rather than any change within a cell; composition is repeatedly found to be a dominant source of variance in blood expression data. Deconvolution can partly recover cell-specific signal, but it is an estimate layered on the same mixed measurement. Circadian time-of-draw. Roughly forty-three percent of protein-coding genes are transcribed rhythmically somewhere in the body, with immune and metabolic genes strongly clock-controlled. Two draws taken hours apart can differ more by the clock than by the disease; without fixed sampling time, diurnal variation is confounded with the contrast of interest. Pre-analytic and ex-vivo induction. RNA is labile, and the draw and its handling perturb the transcriptome they are meant to capture: immediate-early genes (for example FOS and JUN) and handling and hypoxia responses are induced during collection and processing. Tube chemistry, time to stabilization, storage, freeze–thaw, RNA integrity, and globin-transcript contamination all move the numbers. A living cell's respiration is far more robust to this handling than its mRNA. Biological-state confounders. Recent food, exercise, acute infection or vaccination, and — most consequentially — medications remodel the transcriptome; corticosteroids and immunomodulators shift thousands of transcripts and their effects persist. Sex, age, genetic ancestry (through expression quantitative trait loci), and smoking each leave large signatures.

Analytic and statistical fragility. Normalization choices, batch effects confounded with case/control status, and thousands of simultaneous comparisons make false signals easy and reference ranges hard to define; multigene classifier panels tend to overfit their training cohort and are fragile on independent replication. Interpretive and causal limits. Differential expression is correlational. It identifies transcripts that moved — not pathways whose flux moved, and not the direction of causation.

> **Key point.** These are not nuisances to be smoothed over. Cell mixture, the circadian clock, ex-vivo induction, and medication are structural confounders, any of which can produce a convincing "signature" while every sampled cell's mitochondrial function is unchanged.

SECTION FOUR

# The ISRmt: Where Transcript and Function Decouple

The decisive argument for this framework is not a general caveat but a specific mechanism. The mitochondrial integrated stress response (ISRmt) — the state at the center of the CDR / ISRmt model — is fundamentally a translational reprogramming, and it decouples transcript from function by design. When mitochondria signal stress, relays including the OMA1–DELE1–HRI axis converge (with other eIF2α kinases) on phosphorylation of eIF2α. Phosphorylated eIF2α suppresses global cap-dependent translation while selectively permitting translation of stress transcription factors carrying upstream open reading frames — ATF4, ATF5, CHOP. ATF4 then drives transcription of FGF21; CHOP drives GDF15. Three consequences follow, and each one undercuts a transcriptomic readout of mitochondrial state: Global output falls where transcripts do not. Protein synthesis is throttled at the level of translation initiation. A transcriptome, which counts mRNA, cannot see a translational brake: transcripts may be unchanged or rising while protein output — including of respiratory-chain subunits — falls. The transcripts that rise report alarm, not capacity. The ISR transcripts that do increase (ATF4 targets) are the signature of a stress response, not a measure of remaining function. High ISR-target expression means the engine is under strain, not that it is doing more work. Remodeling happens at the layer transcriptomics skips. Function is being reshaped at translation and complex assembly — precisely the steps between transcript and flux that a transcriptome does not observe. So in the exact state this framework targets — CDR2-Mitochondrial physiology, Phenotype B, the ISRmt hub — the transcript-to-function map is not merely weak but inverted: stress transcripts up, functional capacity down. This is why the secreted mitokines GDF15 and FGF21 are useful: as circulating, measurable outputs of the ISRmt, they are a middle path between transcript and flux. But they index that the response is engaged, not how much respiratory reserve remains. To know that, respiration must be measured.

> **Key point.** The ISRmt is a translational reprogramming; the disease state itself decouples transcript from function and inverts their relationship. A transcriptomic panel run on an ISRmt-active cell reports the alarm, not the capacity — and can read “up” while function reads “down.”

SECTION FIVE

# The Seahorse Assay: A Direct Measure of Mitochondrial Function

Extracellular-flux (“Seahorse”) analysis measures, in real time and in living, intact cells, two rates: the oxygen-consumption rate (OCR), a direct proxy for electron-transport-chain flux through oxidative phosphorylation, and the extracellular acidification / proton-efflux rate (ECAR / PER), largely reflecting glycolysis. Rather than

infer function from an upstream correlate, it records the functional output itself. The Mito Stress Test resolves respiration into interpretable components by sequential injection of electrontransport-chain modulators onto the same living cells: Oligomycin inhibits ATP synthase (Complex V); the resulting fall in OCR equals ATP-linked respiration, and the oligomycin-insensitive remainder is proton leak. FCCP, a protonophore, collapses the proton gradient and uncouples respiration, driving the chain to maximal respiration. Rotenone plus antimycin A inhibit Complex I and Complex III, shutting down mitochondrial respiration and revealing the residual non-mitochondrial oxygen consumption. From these maneuvers the assay yields basal respiration, ATP-linked respiration, proton leak, maximal respiration, spare (reserve) respiratory capacity, and non-mitochondrial respiration; a companion XF ATPrate protocol partitions total ATP production into its mitochondrial (mitoATP) and glycolytic (glycoATP) contributions. Each parameter is a measured behavior of the chain, not an inference about it. The reason this is a direct measure is mechanistic. OCR is the integrated output of every upstream layer at once — transcription, translation, post-translational modification, complex assembly, substrate supply, membrane potential, and mtDNA integrity all express themselves in how much oxygen the chain actually consumes. If translation is throttled in the ISRmt, if a Complex I subunit fails to assemble, if membrane potential is low, OCR registers it — regardless of what the transcripts report. The assay reads the answer the transcriptome can only assemble fragments of.

> **Key point.** The Seahorse assay measures the electron-transport chain's actual behavior in living cells and resolves it into functional components. It is the integrated output of all the regulation the transcriptome can only list upstream pieces of — a measurement of function, not a proxy for it.

SECTION SIX

## Spare Respiratory Capacity as Measured Reserve

One parameter deserves separate treatment because it maps directly onto the governing construct of this framework. Spare (reserve) respiratory capacity — maximal respiration minus basal respiration — is the bioenergetic headroom a cell holds in reserve: the additional respiration it can recruit when demand or stress rises. It is the ability to meet a load that exceeds the resting rate. This is reserve, made quantitative — the very quantity the load-over-reserve model treats as decisive. And it exposes a state that resting measures miss entirely. A cell can hold a normal basal respiration — looking unremarkable at rest, and on resting labs — while its spare capacity has collapsed. Such a cell is fine until it is pushed, at which point it has no headroom; this basal-preserved, reserve-depleted pattern is precisely what fails first under load, and it is invisible both to resting biomarkers and to transcript panels. The mapping to the framework's phenotypes is direct. Phenotype B — Drained Battery, CDR2-Mitochondrial — is, in flux terms, a low ATP-linked and low spare-capacity signature; extracellular flux can define Phenotype B directly and grade its depth, rather than infer it. More broadly, the assay converts the framework's central ratio from metaphor into measurement: basal respiration indexes the load currently being handled, spare capacity indexes the reserve available to handle more, and their relationship — load over reserve — is read off a single test.

> **Key point.** Spare respiratory capacity is reserve, measured. It surfaces the basal-normal, re-servedepleted state that resting labs and transcript panels miss, and it renders Phenotype B and the loadover-reserve ratio quantitative rather than inferred.

SECTION SEVEN

# Fair Comparison and the Division of Labor

None of this makes transcriptomics a poor method; it makes it the wrong instrument for one particular job. Its strengths are genuine and largely complementary to flux analysis. Where transcriptomics excels. It is genome-wide and hypothesis-free; it discovers pathways and candidate targets across the whole genome at once; it scales to small samples; and its single-cell and spatial variants partly dissolve the cell-composition confounder. It is the right tool to find a program and generate mechanistic hypotheses to test. Where the Seahorse assay is limited. It is not omniscient. It requires living cells, freshly isolated (PBMCs, platelets, fibroblasts), so viability and logistics matter; measuring ex vivo removes the in-vivo milieu; results depend on seeding density, normalization, and technical execution; and it is mechanismagnostic — it reports the phenotype (how much respiration, how much reserve) but not the lesion (which subunit, which gene). The accessible cells may also not fully mirror the affected tissue. The honest summary is a division of labor, not a winner. Transcriptomics — especially single-cell and spatial — maps the genome-wide terrain and generates hypotheses. Extracellular flux measures whether mito

chondrial function and reserve are actually impaired, and by how much. The circulating ISRmt mitokines, GDF15 and FGF21, sit usefully between them as accessible indices that the response is engaged. For the specific question this framework is built around — staging a patient's bioenergetic state and quantifying reserve — the flux assay is the arbiter and the transcript panel is the map, not the measurement. The field's habit of reading mitochondrial and metabolic "programs" off differential-expression panels should be understood in that light: informative for discovery, but not a substitute for measuring function.

> **Key point.** Transcriptomics maps the genome-wide terrain and generates hypotheses; the Seahorse assay measures the functional truth of the sampled cells. For the question of mitochondrial capacity and reserve, measure it — don't infer it.

SECTION EIGHT

# Summary

Transcriptomics is genome-wide and powerful for discovery, but as a functional readout of mitochondrial state it is indirect: it counts mRNA, which is several regulated steps removed from flux. The central-dogma gap is

quantitative: steady-state mRNA explains only about forty percent of protein variance, and the link loosens further from protein to activity to flux; mtDNA copy number and heteroplasmy are largely invisible to nuclear RNA-seq. Blood transcriptomics carries structural confounders — cell-type composition (the dominant one), circadian time-of-draw (about forty-three percent of genes are rhythmic), ex-vivo induction during collection, medication and state effects, and analytic fragility — any of which can manufacture a signature with no change in cellular function. The ISRmt, the target state, is a translational reprogramming: eIF2α phosphorylation suppresses global translation while selectively translating ATF4 and CHOP (and their mitokines FGF21 and GDF15). Transcript and function decouple and can invert — stress transcripts up, capacity down. The Seahorse assay measures oxygen consumption in living cells and resolves it (oligomycin, FCCP, rotenone/antimycin A) into basal, ATP-linked, proton-leak, maximal, spare, and non-mitochondrial respiration — the integrated output of all upstream regulation. Spare respiratory capacity is reserve made quantitative; it exposes the basal-normal, reserve-depleted state and renders Phenotype B and the load-over-reserve ratio measurable. Use transcriptomics to discover and hypothesize; use extracellular flux to measure mitochondrial function and reserve; use GDF15 / FGF21 as accessible indices that the ISRmt is engaged. For capacity and reserve, measure — don't infer.

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*Organized by section. Entries should be reconciled against the master citation apparatus during typesetting.*

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