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From Faulty Biochemical Assumptions to Poor Diagnostic Performance: Reflections on the Failure of DNA Microarrays to Become a Reliable Clinical Diagnostic Tool

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Author's contribution

This whole work was carried out by author SR.

Opinion Article

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ABSTRACT

Since their inception about two decades ago, DNA microarrays have been considered as a great hope in translational research and personalized medicine. Although DNA microarrays for gene expression profiling proved to be an indispensable tool in the laboratory settings, their applications as an instrument for clinical diagnostics have not yet produced tangible results. In this paper, we convey the idea that, apart from notoriously poor reproducibility and complexities of experimental validations, there exist other reasons hindering clinical application of DNA microarrays. These reasons are rooted in the very core of the DNA microarrays methodology, that is, in faulty biochemical assumptions underlying microarray measurements. A key premise the microarray measurements are based on is that mRNA abundances harvested from the eukaryotic cytoplasm are indicative of the activity levels of corresponding genes. There are at least two reasons why this premise is questionable. First, each transcription is supported by a number of transcription factors expressed by many genes. Due to this reason, relations between the transcription rates of genes and the mRNA abundances are the 'many-to-one', not the 'one-to-one'; therefore, abnormality in a certain mRNA abundance does not unequivocally indicate abnormality of the gene bearing its complimentary code. Second, mRNA copy numbers in cytoplasm are regulated by a number of epigenetic factors among which the post-transcriptional mRNA stability is of primary importance. Abnormal concentration of certain mRNA may result from deviant

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mRNA stability, thus mimicking, but having nothing to do with, presumed abnormality in transcription rates of corresponding genes. An instrument built upon so poorly understood biochemical basis can hardly serve as a reliable tool in the delicate task of diagnosis of human disease in clinical settings.

Keywords: DNA microarrays; post-transcriptional mRNA stability; genetic regulatory networks; mRNA abundance; transcription rate; gene activity; clinical applications.

1. INTRODUCTION

1.1 Clinical Promise and Pitfalls of the DNA Microarray Measurements

A common concern in microarray data analysis is poor reproducibility. In the editorial [1] preceding the report summarizing the large-scale Microarray Quality Control Project [2], this aspect of microarray measurements has been characterized as follows: *"Doubts linger about the reproducibility of microarray experiments at different sites, the comparability of results on different platforms and even the variability of microarray results in the same laboratory. After 15 years of research and development, broad consensus is still lacking concerning best practice not only for experimental design and sample preparation, but also for data acquisition, statistical analysis and interpretation... Clearly, microarrays have a long way to go before they can be used to support regulatory decision-making or accurate and consistent prediction of patient outcomes in the clinic."*

Since inception, DNA microarrays for gene expression profiling (to be distinguished from the ones for microbial and viral detection through DNA hybridization [3-6]) have been considered as a great hope in the bench-to-bedside translational research. These hopes have been expressly formulated in a number of early papers [7-10] on microarrays. For example, we find in [8]: *"Measurements of genomic and proteomic alterations may be used to establish more specific diagnoses, to select optimal therapies and to monitor patients' response to therapies, and for a broad variety of diseases, most notably cancer."* However, it should be noted that from the very outset, the journey of the "array of hope" [10] from experimental laboratory towards clinical application was not expected to be easy and self-evident, especially in the domain of cancer diagnostics and treatment. For example, Masters & Lakhani wrote: *"Analysis of gene expression using DNA microarrays is unlikely to replace histopathology as the prime indicator of prognosis"* [11].

FDA identified the microarrays technology as crucial in advancing medical product development and personalized medicine. As a first modest sign of success on this thorny path, the microarray-based prognostic assay designed to individualize treatment for patients with breast cancer, called Mamma Print (Agendia BV, The Netherlands), has been commercially developed and approved for clinical testing [12]. Genomic arrays found their way into prenatal diagnostics: in Belgium, a national consensus between national Centers for Medical Genetics has been reached regarding practical aspects of using and reporting DNA microarray findings [13]. However, these and similar results, being undoubtedly promising, are disproportionally scanty as compared to the monumental efforts and massive expenses committed to the development of this technology.

High stakes and hopes attached to microarrays are in stark contrast to the chorus of skeptical opinions regarding microarray diagnostic capabilities and technical performance. Thus J. Ioannidis, in his paper with a telling title "Microarrays and molecular research: noise discovery?" [14], writes: *"The promise of microarrays has been of apocalyptic dimensions...All diseases are to be redefined, all human suffering reduced to gene-expression profiles. Cancer has been the most common early target of this revolution, and publications in the most prestigious journals have heralded the discovery of molecular signatures conferring different outcomes and requiring different treatments. Yet, on close scrutiny, in five of the seven largest studies on cancer prognosis, this technology performs no better than flipping a coin. The other two studies barely beat horoscopes".* This austere opinion is echoed in [15]: *"The potential advantages of improving tumor classification by expression profiling has been central for several large-scale breast cancer studies that have reported identification of signature gene lists with potential for prediction of clinical outcome over the past few years. The microarrays used in different studies generally have several thousand genes in common and the underlying principles of the measurement technologies are the same. The most striking finding when comparing the signature lists is the virtually complete lack of agreement in the included genes."*

Detailed evaluation of the state-of-the-art in microarray technology presented in [2] is summarized as follows: *"Profound problems in data quality have been observed from analyzing published data sets, and many laboratories have been struggling with technical troubleshooting rather than generating reliable data of scientific significance...These fundamental issues must be adequately addressed before microarray technology can be transformed from a research tool to clinical practices."*

In the above quoted report [2], all the *profound problems* of microarrays are categorized in four big classes: Technical (microarray manufacturing, sample collection, RNA extraction, cDNA and cRNA synthesis, fluorescent labeling and hybridization); instrumental (laser intensity, scanner calibration, image acquisition and spot quantification); computational (data preprocessing, normalization, statistical analysis of differential expression); and interpretive (biologic reasoning, pathway analysis, bioinformatics tools). The authors point out that *"A single hidden, uncontrolled factor may completely negate an experiment."* Obviously, in clinical settings the cost of such a *single, uncontrolled factor* may be much higher than in the laboratory and may lead to wrong diagnosis with potentially harmful consequences for patient-related decision making.

In-depth review of the current status and impediments in development of the microarray-based diagnostic techniques has been recently presented in the comprehensive report "Evolution of Translational Omics. Lessons Learned and the Path Forward. [16]" In this voluminous 500+ pages document, authored by a large group of distinguished scientists and approved by the Institute of Medicine of the National Academies, current status of the omics technologies in general, and microarray-based assays in particular, are characterized as follows:

The frequent lack of a clear biological rationale further distinguishes omics-based tests from most other clinical laboratory tests based on a single analyte. The biological rationale behind a single-analyte test is frequently quite evident: The test is useful because the gene, RNA, protein, or metabolite plays an understood role in the disease pathology or other biological processes under investigation. In contrast, the biological rationale for the set of biomarkers in an omics-based test frequently is not well-defined scientifically. This difference puts an

additional burden on the statisticians and bioinformatics experts involved in test validation to ensure that the biological data and computational model are scientifically sound.

As seen from the critical evaluations quoted above, at this point, the foundations of the microarray-based diagnostic techniques should not be considered as solidly constructed and satisfactorily understood, hence further inquiry into this subject is well justified. It should be also noted that in this inquiry additional burden should be put not only on statisticians and bioinformaticians (as mentioned in the excerpt above), but also on biochemists – to better understand molecular biology of gene expression, on physicists – to improve measurement performance, on systems biologists – to create an adequate systemic view of parameters being measured, on computational scientists – to create appropriate mathematical models of the processes underlying microarray measurements.

In this paper, two aspects of the DNA microarray methodological weakness are analyzed. First, strong interactions within intracellular biomolecular networks make it difficult, if possible at all, to build a bridge between individual mRNA abundances evaluated by microarrays and structural fidelity of the corresponding genes. Second, attention is drawn to the fact that in the absence of readily available information regarding the post-transcriptional mRNA stabilities it is highly problematic to correctly evaluate the level of gene activity from the relative mRNA abundances.

In the experimental laboratory settings, the above outlined drawbacks of DNA microarrays may be effectively compensated by a number of complimentary (generally more advanced, more expensive and more laborious) techniques such as RT-PCR, Southern blot, SAGE and others. It is not to mention routine validation of any important finding through replication in the same or in a different laboratory. All this luxury of unlimited experimentation is not appropriate in clinical settings. To avoid misconception, in this paper under the words "clinical settings" we assume the situation when an *individual* patient is offered a microarray-based diagnostic test, much in the same manner as other in- or outpatient diagnostic tests, such as PSA, or PAP smear, or ECG, or colonoscopy, or strep test, to name just a few. A clinical diagnostic test should satisfy a number of obvious requirements. First, it should be self-sufficient, that is capable of resolving a diagnostic inquiry by itself, without extensive support of other techniques. Second, a clinical diagnostic test should have high level of specificity and sensitivity; these properties should be established, of course, in extensive preclinical testing. At last, but not the least, it should be cost-effective; needless to say that health insurance policies are not designed to fund extensive laboratory research in support of individual diagnostic decisions. The point we attempt to make in this paragraph is that even if a certain microarray-based technique has proven to be highly efficient in the domain of scientific discovery, it still may fall short of being an acceptable diagnostic tool in clinical settings.

2. MICROARRAYS ANALYZE THE TRANSCRIPTOME, NOT THE GENOME

Major steps in the microarray-based diagnostics may be schematically represented as follows. First, mRNA molecules are harvested from the cytoplasm of patient's cells and their abundances are compared to those of normal cells. Abnormalities in the mRNA abundances are evaluated on gene-by-gene basis and ascribed to abnormal functionalities, presumably because of mutations, of the genes from which these particular mRNAs are transcribed. The differential expression levels are treated as biomarkers and serve as goalposts for pharmaceutical industry in developing the drugs seeking to compensate for genetic disease through therapeutic modifications of metabolic pathways. In the nutshell, microarrays

analyze the abnormalities in transcriptome whereas cures target to compensate the abnormalities in the genome. Obviously, the mRNAs themselves cannot be a target of therapeutic intervention. As a close analogy, if the PSA is found to be elevated in a patient then it would not make much sense to design a drug for decreasing the PSA level. Instead, the underlying reason, that is, the possible prostate cancer, has to be the target of a therapy. Imbalances in the mRNA abundances serve only as the messengers of genetic abnormality; and as an old adage says, "don't shoot the messenger." The goal of therapeutic intervention is to correct for abnormality of genes, not of mRNAs, most prominently through modification of metabolic pathways involving the genes' transcription factors.

Since their introduction into laboratory practice, the microarray measurements have been termed gene expression profiling, and this term has been used interchangeably with transcription profiling. When using such terminology, it is assumed to be self-evident that through the measurements of intracellular mRNA abundances one may come to some conclusions regarding the status and/or activity of the genes whose complimentary codes these mRNAs bear. However, as is well known, each transcription event is supported by a team of transcription factors (TF) which in turn are nothing else but the proteins expressed by other genes (e.g., [17].) This means that any gene-specific mRNA molecule is in a sense a product of teamwork of cooperating genes, and in which the gene actually responsible for the synthesis of specific mRNA may not even play a dominant role. Due to this essential reason, the relations between the genes and mRNAs may be characterized as a 'many-to-one' connection, and not as a 'one-to-one' connection. Despite this rather obvious argument, numerous examples can be found in the literature in which the microarray fluorescent intensities are directly used for fitting the models of *genetic* regulatory networks [18-26]. These examples clearly indicate that there is a widespread tendency to assume that measurements of the mRNA abundances represent a reasonably solid basis for making the inferences regarding transcriptional activity of corresponding parent genes. In reality, mRNA cytoplasmic abundances may only produce some fairly fuzzy correlative evidence regarding the functional veracities of the genes from which the mRNAs are transcribed. In no way, however, they may serve (without support of more sophisticated techniques) as direct unequivocal indicators regarding the genes' functionality and integrity of their genetic codes.

From the systemic viewpoint, intracellular biochemical machinery is a high-dimensional highly nonlinear system in which interactions are governed by the laws of chemical kinetics. A brief term for denoting such systems is *biochemical networks*. A fundamental feature of all the networks is that no event within them can occur in isolation, independently of other events. Figuratively speaking, each individual event creates a domino-effect of events propagating throughout the system. Genetic regulatory networks represent a perfect example of biochemical networks with tight interdependencies between individual gene expressions. For example, if a transcription factor originated from the gene-A failed to report to the regulatory site of the gene-B, then the latter will be halted thus mimicking its low or zero activity. Harvesting the mRNA-B using microarrays will show its low abundance, and naïve direct interpretation of the microarray data would implicate gene-B in abnormal functionality, perhaps in presence of some mutations. Based on this finding, pharmaceutical industry may find justification for launching development of a drug with the gene-B being a target for therapeutic intervention. In fact, however, it may be the gene-A to blame for the failure of the gene-B. Things, of course, are much more complex in multi-gene regulatory networks. This is because typically *dozens* of TFs are required by each gene to produce a single mRNA, and therefore dozens of genes may be responsible for the failure of the gene of interest. In somewhat loose terms, it may be said that a network acts as a whole and reacts as a whole. This tight interconnectedness makes it difficult, both technically and

conceptually, to analyze the observational and experimental data on gene-by-gene basis. In particular, the mRNA abundances, to a large extent, are the products of the system's collective behavior, rather than of activity of individual parent genes. Lack of clarity, if not to say outright confusion, in the interpretation of the mRNA abundances may have far reaching detrimental consequences.

In order to untangle complex relationships between observed behavior of the transcriptome (that is, totality of all the mRNAs) and the genome (that is, totality of all the genes), some basic quantitative measures should be introduced. Detailed discussions of all the pertinent questions have been given in a number of previous works by this author [27-32]; here we provide only a brief sketch of the basic concepts.

According to the *Central Dogma* in molecular biology [33], there is a unidirectional flow of biological information from genes to proteins, with the mRNA being an intermediary. Per copying from genetic code by RNA Polymerase (transcription) and prior to protein synthesis by ribosomes (translation), the mRNA molecules undergo numerous transformations, some sequence-specific, some not. The key steps include export of nascent mRNA to cytoplasm, removal of non-coding regions of mRNA (splicing), maturation, editing, and other processes preceding binding of mRNA molecules to the ribosomal sites [33,34]. It is within this time span between transcription and translation, the mRNA is being isolated for preparation of the microarray assays.

For quantitative description of transcription, two concepts should be introduced, that is, transcription level (TL) and transcription rate (TR). TL is the number of the mRNA copies *present* in the cell at a certain moment of time. TR is the number of transcripts *produced* within the cell per unit of time. Obviously, it is the TR that is a direct characterization of the gene's activity. The higher TR, the more transcripts are produced per unit of time, hence the more active is the gene. In contrast, it is the TL that is being registered in microarray measurements. The higher TL the greater is the fluorescent intensity of the cDNA spot in the microarray, the fact which presumably indicates higher mRNA abundance.

Mathematically, the roles of TR and TL are expressed by the equation

$$\frac{d}{dt}TL(t) = TR(t) - \frac{1}{\tau}TL(t) \tag{1}$$

Where τ is the characteristic time of mRNA degradation. All the quantities above are mRNA- and gene-specific. Under the term degradation, we mean all the biochemical processes contributing to mRNA disappearance from the cytoplasm; among them the most important are the interactions with ribosomes leading to protein synthesis.

As seen from the above definitions, there are no and cannot be any direct relationships between TLs and TRs; this would be true even in a simple hypothetical system containing only one gene and one mRNA. It is not out of place to mention that TL and TR have different physical dimensions (i.e., copy number and copy number per second, respectively). According to laws of dimensions, in order to establish any sort of linear relationships between TL and TR, knowledge of some parameter with the dimension of time would be required. This parameter should be either known a priori or derived from the data; however, in microarray measurements only one variable can be evaluated, and this variable is TL.

An attempt to evaluate experimentally possible connection (or lack thereof) between the TL and TR has been undertaken in [35]. In this time-course experiment, the TLs (mRNA copy numbers) and TRs (rates of elongation) have been measured simultaneously in budding yeast using sophisticated laboratory technique called Genomic Run-On. It was found that from a total of 5,500 $TR \& TL$ time-series pairs, about half turned out to be uncorrelated with each other. Partial explanation to this phenomenon has been given in [27,28] by this author. Generally, the results of this remarkable experiment demonstrate that the are no direct relations between transcription rates and cytoplasmic mRNA abundances.

Simple example may be helpful in elucidating the $TL-TR$ relationships. The difference between them is similar to that between someone's income and his/her account balance. Even complete knowledge of the account balance generates no information regarding the income, and vice versa. Likewise, complete knowledge of the transcription levels creates no information regarding transcription rates. In other words, measurements of the mRNA abundances are not directly representative of gene activity.

3. ELEPHANT IN THE ROOM: POST-TRANSCRIPTIONAL MRNA STABILITY

Cytoplasmic abundance of the mRNA comes to existence as a result of establishing dynamic equilibrium between mRNA production and degradation. As seen from equation (1), if such equilibrium indeed exists (which is not guaranteed in high dimensional biochemical networks [28,31]) then TL and TR are connected by linear relation

$$TR = \frac{1}{\tau} TL, \tag{2}$$

Where τ is characteristic time of mRNA degradation. The value of this parameter depends on a number of biochemical processes in cytoplasm. In the context of microarray measurements, two circumstances should be emphasized. First, τ is an epigenetic quantity; as such it does not depend on TR of the parent gene. Second, evaluation of this quantity is not a part of standard microarray protocol. Simply put, it is unknown, and therefore cannot be included in routine testing in clinical settings. Inclusion of the direct evaluation of τ into the microarray protocol (say, in the manner of experiments [35]) would launch the price of clinical diagnostic to astronomical heights because no routine, well established and cost effective methodologies for doing this are seen anywhere close on the horizon.

Traditionally, the rate of biochemical degradation is measured by the quantity called *half-life*, $t_{1/2}$, that is, the time required for a concentration (or a copy number) to drop to the half of its initial level. (In linear systems, when the characterization in terms of a single degradation time is justified, there is a simple relation between the half-lives and degradation times: $t_{1/2} = \tau \ln 2$) In the context of microarray measurements, the crucial questions are whether or not the degradation rates of different mRNAs are substantially different, and if they are then whether or not they are sequence-specific. Significance of these questions is in the fact that if the half-lives were approximately of the same order of magnitude for all the mRNAs then they could be measured by a single non-specific transcriptome-wide constant. Then, due to equation (2), absence of information regarding τ would not substantially hinder

differential expression testing. In order to figure out whether or not such a scenario is indeed possible, we review some basic empirical facts pertaining to the mRNA degradation.

It has been shown in [36] that in mouse embryonic stem cells, the mRNA half-lives vary within the range from 2 to 24 hours and beyond. In yeast, according to [37], mRNA half lives range from 3 min to 1.5 hours. The study of time-course kinetics of ~1500 mRNAs in microbial cells has revealed that half-lives vary from less than 30 seconds to more than 20 minutes [38]. It is not yet well known what biochemical factors influence degradation rates. It is known, however, that the mRNA decay rates are not sequence-specific, therefore, there is no connection between them and the complimentary genetic code they bear. Rather, half-lives are dependent on the mRNA's length and some structural properties such as the number of exon junctions per open reading frame [36]. Wide variations of mRNA half-lives ranging from several minutes to several hours in human T lymphocytes have been also reported in [39]. To make things even more tangled, the mRNAs were found to have different stabilities at various cell stages and at various developmental stages of the organism. As a drastic example, the authors [40] reported that immunoglobulin p mRNA is at least sixfold (!) more stable in the late versus early stage of B cells. Broad study of factors influencing mRNA stability has been undertaken in [37]. These authors came to an important conclusion that epigenetic control of the mRNA decay rates is a fundamental feature of gene expression *machinery*. We conclude this brief overview by the proposition that τ generally mRNA-specific and cannot be excluded from consideration in differential expression testing, thus being always present as a strong confounder in the microarray measurements. A stable transcript may exist in high concentrations in cytoplasm regardless the status of the parent gene. An unstable transcript disappears quickly per entering the cytoplasm thus wiping out the gene's representation in the transcriptome. Traditional interpretation of microarray data flatly ignores this important, and completely epigenetic, source of the mRNA variations. We come again to the same conclusion as before: the microarray measurements produce information about the transcriptome, not about the genome. There no and cannot be any direct relations between them. But ultimately, it is the genome, not the transcriptome, that is supposed to be a target of therapeutic intervention. Microarrays may help in the task of experimentation with molecular mechanisms of gene expression, but are intrinsically incapable to serve as a definitive diagnostic test.

4. EXPERIMENTAL LABORATORY AND CLINICAL DIAGNOSTICS: DIFFERENT WORLDS, DIFFERENT REQUIREMENTS

In experimental laboratory settings, microarrays are usually used as an instrument in ongoing research within a wider experimental environment. To a large extent, microarrays are used for preliminary evaluation of genomic irregularities and for hypotheses generation. Should some promising leads surface in the differential expression analyses, they may be always rechecked through replications. If the effect is found to be stable and statistically significant, then usually more accurate (Albeit often more expensive) methods are available for validation (eg, RT-PCR). Research process is an open-ended enterprise in which reliability of discovery is of higher priority as compared to urgency in arriving at final result. Deliberations, discussions, discrepancies in interpretations are typical for research environment. A researcher in scientific laboratory settings bears no direct responsibility for the life and health of any particular patient. The ultimate outcome of research process is usually expressed in the form of scientific publication, or report, or policy-making recommendation.

The world of clinical diagnostics is different. The goal of clinical tests is to produce a one-stop evaluation of the patient's status and to provide a physician with a key information assisting in therapeutic decisions. In this sense, a microarray assay should play a role analogous to other clinical tests, such as, say, PAP smear, PSA, urine test or blood cells count. Clinical test is supposed to adhere a strict standardized protocol and to be calibrated in a certified laboratory. Obviously, bedside is not an appropriate place for resolution of scientific controversies and/or clashes of methodological interpretations.

Since time and money are of the essence in clinical settings, no further experimentations, especially involving costly equipment and supervision of highly trained professionals, could be affordable. Sense of urgency and responsibility for the patient's health and life are always present in clinical practice. Contrary to the experimental laboratory settings, not a scientific publication, but a definitive diagnose and recommendation for treatment are expected to be an ultimate goal in clinical settings. Needless to mention that any clinical test is supposed to be FDA approved.

Considerations offered in this paper are not, of course, a final verdict to the microarray-based clinical diagnostics. They are only supposed to outline the status-quo in this extraordinarily important domain of bio-science and bio-technology. Currently, great efforts are being undertaken to avoid the drawbacks of existing techniques, to create more sophisticated microarrays, to standardize protocols, to miniaturize and make cheaper the techniques that are currently considered prohibitively expensive. This paper is an attempt to attract attention to some pitfalls of existing DNA microarrays; hopefully, it can help to circumvent them in future.

5. CONCLUSION

DNA microarray proved to be an indispensable tool in scientific and laboratory settings. Following the leads provided by DNA microarrays, innumerable discoveries have been made in experimental biology, despite all the complexities and unresolved issues.

DNA microarrays provide important leads for follow-up studies and often justify application of more advanced technologies such as PCR, ChIP, protein mass spectrometry, reporter plasmid analysis, Southern blot, Northern blot, SAGE and direct DNA sequencing. However, clinical practice requires definitive and cost-effective diagnoses, not just the leads for further experimentation. Microarrays cannot provide such definitive conclusions. It is the author's opinion that there is still long way to go until this becomes possible. Biochemical fundamentals of microarray measurements are still poorly understood and a number of confounders with wide spectrum of uncontrollable variations remain out of scope of routine protocols. In conclusion, the methodology based on so shaky grounds requires further investigation and refinements before it may reach the status of reliable diagnostic tool in clinical settings.

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ETHICAL APPROVAL

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Author has declared that no competing interests exist

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