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Machine learning-based metabolic imaging: A bridge between *in vitro* models and clinical applications

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Summary. — A multiparametric approach is necessary to understand the intricate processes driving cellular physiological and pathological states. Current developments in the field of optical microscopy offer a deeper understanding in the evaluation of minute molecular changes, together with an even greater volume of biological data. Among these developments is functional metabolic imaging, a field that integrates molecular biology and *in vivo* imaging and sheds light on a number of metabolic processes that are crucial to cell survival. A few inherent limitations of the frequently used methods can also be solved by using probes that modify the picture in response to chemical changes occurring in the region of interest. To decode the extensive useful biological material that is concealed in pictures, more potent image analysis techniques are needed in addition to advancements in the image capture process. Many machine learning (ML)-based techniques have recently been developed to achieve this goal. After providing a brief overview of metabolic and multiparametric imaging and the ML-based approaches that we are interested in, I will present different applications that I have developed to enable a more in-depth investigation of lipid turnover and membrane phase states, which are necessary to understand the functional and structural changes occurring in the disease.

1. – Introduction

All living systems depend on a steady stream of metabolic energy. This energy is characterized by a series of intricate chemical processes that control a wide range of biological functions, from the maintenance of intracellular energy homeostasis to intercellular signaling pathways [1]. In this sense, metabolism is commonly understood to refer to a group of metabolic processes that either produce or use energy in living things [2]. Basic metabolism can be reduced to the metabolic processes involving the most common nutrients, such as amino acids, fatty acids, and carbohydrates, which are required for human energy homeostasis and macromolecule creation [3]. It is possible

to differentiate three paths within it: the creation of simple molecules or their polymerization into more complex macromolecules (anabolism); the breakdown of molecules to produce energy (catabolism); and the elimination of harmful byproducts produced by other processes (waste product disposal). Between 1920 and 1960, most of the processes that make up the metabolic network were defined, including glycolysis, respiration (Warburg) [4], the tricarboxylic acid (TCA) [5] and urea (Krebs) cycles [6], and oxidative phosphorylation [7] and this period of time was driven by the realization that metabolic perturbations accompany common human diseases. However, the emergence of new fields of biological examination, with a focus on study into the genetic and molecular basis of diseases including, among others, diabetes [8] and neurodegeneration [9], is accompanied by a simultaneous slowing after an initial period. However, although gene expression, signal transduction, cell differentiation, and other processes are not typically thought of in terms of bioenergetic or metabolic processes, the intense multidisciplinary research work has made many common diseases now recognized in terms of inherited or somatic mutations that disrupt these processes [10]. For a better understanding of this idea, we can imagine cell metabolism as a network of public transportation, with each line representing a distinct metabolic pathway, each station analogous to a molecule, and each connection denoting a specific reaction, resulting in a close relationship between each different metabolic pathway [11]. This explains why any alteration in cellular physiology is linked to changes in a particular area of metabolism, and vice versa, and why any perturbation in one node of metabolism causes a global response in the organisms. So, it is reasonable to suppose that any disease's onset can be linked to a particular biochemical signature that can be found and identified by contemporary investigative techniques. Among the different metabolic pathways, lipid metabolism deserves specific consideration. At the cellular level, lipids play three fundamental roles, acting as an energy reserve, components of membranes, and signaling molecules [12]. Each of these tasks has a clear relationship and impact on the etiology of different diseases, both metabolic and non-metabolic. Changes in membrane structure, which affect its fluidity, can alter transport mechanisms [13], and particular fatty acids can trigger inflammatory pathways [14]. All these effects of storage imbalances can potentially result in the creation of harmful aggregates within cells [15]. The direct involvement in the pathogenesis of different diseases demonstrates how lipid metabolism research can serve as a potential biomarker for the early detection of these pathologies, also emphasizing the urgent need for a method that can monitor intracellular lipid management in real time and with high spatial resolution. Among the different metabolic diseases, diabetes mellitus (DM) has seen a significant increase in incidence over the past few decades. In Italy alone, 3.27 million people claim to have the condition (5.4%), but at least one million cases go undiagnosed, and four million people are at high risk of getting it [16]. Not only does this influence society, but it also implies enormous expenditures for the healthcare system —both direct costs (drugs, hospitalisation, and help) and indirect costs (missed productivity, costs for the social security system). From this angle, it is obvious that measures that focus on early diagnosis, efficient treatment, but also, and most importantly, prevention, are needed [17]. We therefore aim to develop tools that allow exploring the relationship between metabolic impairments and the development of vascular complications associated with type 2 diabetes, investigating changes in membrane fluidity in distinct cellular systems through machine-learning–based metabolic imaging.

2. – Multiparametric metabolic imaging

Metabolic imaging is a field that integrates molecular biology with *in vivo* imaging methods to visualize endogenous molecules and supramolecular features, offering a complete window on all the metabolic processes that are necessary for cell survival [18]. Thanks to a number of increasingly powerful and advanced techniques, metabolic imaging allows a multiparametric approach, which consists in assigning to each pixel, as a label, different measured quantities, thus providing several distinct information. Currently, the development of a wide range of imaging techniques, as well as the refinement of existing ones, allows for the capture of multiparametric information on the biophysical state of membranes and organelles, allowing for the competent differentiation of many phases. This has the potential to enable real-time sub-micrometric investigations of cell membrane phase transitions in living cells and tissues under various physiological and pathological situations. Nevertheless, multiparametric imaging is constrained by the need for strong instruments to analyze vast amounts of data in order to extract and decode biological information. In this regard, contemporary technologies based on machine learning and artificial intelligence provide a useful technique to automate analysis through training procedures. This eliminates the need for human data processing changes, making these solutions more resilient than traditional approaches and notably more adaptable in solving complicated multidimensional data analysis problems. Furthermore, the application of these methodologies allows us to visualize metabolic features that conventional methods would not allow us to investigate.

3. – Introduction of artificial intelligence and machine-learning–based tools

Arthur Samuel created the term "Machine Learning" in 1959 to describe the field of artificial intelligence that uses statistical approaches to increase an algorithm's ability in finding well-defined structures (*i.e.*, patterns) in data [19]. After acquiring experience on a set of learning data, the basic goal of machine learning is for a machine to be able to generalize from its own experience, *i.e.*, to accurately accomplish novel tasks that it has never experienced. Several categories of machine learning approaches can be distinguished based on the sort of input on which the learning system is based.

In the instance of supervised learning, the computer is shown examples of inputs and their desired outputs with the goal of learning a general rule that permits the inputs to be mapped into the outputs. Under this category, we separate the classification, in which the inputs are divided into two or more classes and the learning system must generate a model capable of assigning one or more classes from those accessible to an input. Instead, in the unsupervised approach, the computer is given simply input data and no predicted output, with the goal of detecting and learning some structure in the input data. Unsupervised techniques include clustering, which, like classification, divides a set of data into groups that are not known *a priori*.

These methodologies, relying on powerful artificial intelligence-based algorithms, allow decrypting biological features and correlations concealed in microscopy images and numerical data, bringing up a wide range of application possibilities. To my purposes, I will now focus on and discuss two approaches to explore lipid turnover, membrane phase state, and remodeling in living cells, both of which have relatively validated applications. These methodologies are critical for thoroughly assessing the metabolic changes that occur in pathological situations, highlighting the underlying mechanisms of numerous metabolic illnesses including, among others, diabetic retinopathy (DR).

4. – Application of machine-learning–based tools to monitor metabolic changes in different systems

4˙1. *Machine-learning assisted confocal imaging for the analysis of lipid metabolism variations during cellular differentiation.* – To explore intracellular lipid turnover, the first strategy employs supervised pixel classification. To categorize nonpolar compartments in cells, this strategy combines machine learning (ML)-based methods and spectral phasor analysis [20].

As previously stated, lipid metabolism is critical in many cells because it provides both energy and essential chemicals for membrane production and signaling pathways. Since any disruption in lipid storage or subsequent consumption can indeed disrupt the lipid homeostasis of the entire system, real-time mapping with high spatial and temporal resolution of the overall process of lipid aggregate formation, from initial synthesis and accumulation in aggregation sites to final coalescence and growth of lipid droplets (LD), is critical for identifying any potential metabolic imbalance and predicting and counteracting its progression. However, techniques currently used to study this FA metabolic route have several disadvantages, being intrusive and disruptive to samples, in addition to having poor resolution and selectivity. Several fluorescence-based methods have thus been developed to allow real-time monitoring: fluorescent vital stain, such as Nile Red [21] and BODIPY [22] have been used to detect intracellular LD formation and growth in cells, tissues, and living animals [23, 24]. Aside from detecting LD formation, our lab has devised a fluorescent approach for real-time determination of the general level of activation of FA metabolism acquired by measuring the microfluidity of the membrane environment [25, 26]. However, all these techniques allow for the identification and measurement of LD formation and size, not providing information on the first phase of FA conversion into triglycerides (TAG) and cholesterol in cholesteryl esters (CE) in organelles other than LD. In this context, I have developed a new method, which is fully described in [27], that allows not only to detect the number and size of lipid droplets with high selectivity, but also to localize and quantify the intracellular deposits of TAG and CE, allowing a deeper real-time monitoring of the entire process of lipid turnover in living cells.

The developed polarity-driven segmentation (PDS) has been performed using a commercial confocal spectral imaging setup, which acquires the fluorescence emission spectrum for each pixel, and data have been analyzed through the spectral phasor approach. We used PC12 cells as a model cell line to investigate changes in lipid metabolism induced by Nerve Growth Factor (NGF) therapy, which causes cell differentiation into a neuronal phenotype. Our results revealed a different spatial distribution between undifferentiated and differentiated cells, with the latter characterized by a more scattered distribution throughout the cytoplasm. By quantifying the fraction of LD and TAG-CE sites, we observed the NGF-treated cells are characterized by a higher fraction of TAG-CE depots with respect to undifferentiated cells. The major improvement of the suggested methodology, besides providing a finer detection of the LD core, is the detection of TAG-CE stored in regions other than LD, which may constitute the formation sites of LD on the ER. In recent years, there has been a surge of interest in the involvement of lipid metabolism in signaling pathways within the central nervous system (CNS) [28] and in the start and progression of many diseases. Indeed, neurons have a high lipid content, and lipids are involved in a variety of cellular functions. Without a doubt, their significance stems primarily from the role they play in biological membranes, their bioactive contribution as messengers in intra- and inter-cellular signaling,

and their position as energy suppliers [29]. The application of this approach on NGF-treated PC cells, used as a model to explore the lipid metabolic modifications involved in nerve cell growth, revealed a considerable rise in the fraction of LD core in differentiated cells, with a fraction more than 5-fold higher with respect to undifferentiated cells [27]. Overall, these findings suggest that an increase in lipid turnover supports the cell energy demand required to sustain the morphological and functional reorganization of cells during differentiation process [30]. In addition, we were able to provide insights on the distinct spatial distribution of intracellular TAG-CE deposits, highlighting the metabolic reprogramming of differentiated PC12 cells. The domain-specific architecture observed in NGF-treated cells may represent a differentiation-induced membrane remodeling process, which requires considerable amounts of fatty acids as membrane building blocks for axonal elongation and secretory vesicle production [30]. Importantly, the role of lipid metabolism in the initiation and progression of neurodegenerative illnesses [31], such as Age-related Macular Degeneration (AMD) and diabetic retinopathy (DR), as well as the presence of toxic lipid aggregates in neuronal and glial cells *in vitro*, has recently been documented [32]. Precision management of the entire TAG-CE turnover, from the early stages of FA metabolism to the final synthesis of LD, is thus required to monitor the evolution of these diseases and test the efficacy of interventions. In this context, the ability to detect LD core and identify early locations of non-polar lipid aggregation can be a useful tool in the prevention and therapy of a variety of diseases, including diabetes-induced blood-retinal barrier disruption. Moreover, in addition to providing the ability to act through metabolic-based approaches aimed at lowering glucolipotoxicity induced by ectopic fat deposits, these approaches can also be utilized to further monitor the success of applied treatments.

4˙2. *Unsupervised clustering of multiparametric fluorescent images extends the spectrum of detectable cell membrane phases with sub-micrometric resolution.* – The membrane phase dynamic equilibrium reflects changes in the lipid and protein composition, as imposed by dietary, environmental, and cellular conditions, making its maintenance a prerequisite for proper membrane function, cell growth and division [33]. Solvatochromic probes, being characterized by an emission shift when the hydration level of the membrane environment increases, have been widely used to distinguish between solid-ordered and liquid-disordered phases in artificial membrane bilayers. However, this emission shift is currently limited in unraveling the broad spectrum of membrane phases of natural cell membranes and their spatial organization.

In this perspective, we proposed a clustering-based analysis that, leveraging on the multiparametric content of spectrally resolved lifetime images, allowed us to classify through an unsupervised learning approach multiple membrane phases with sub-micrometric resolution. This method extends the spectrum of detectable membrane phases allowing to dissect and characterize up to six different phases, and to study real-time phase transitions in cultured cells and tissues undergoing different treatments. This is crucial since the membrane phase separation is involved in several mechanisms, playing a pivotal role in the potency of ligand binding to membrane receptors, on direct cell-cell interaction and on the modulation of the activity of membrane enzymes, receptors, channels, and transporters, and its alteration has been associated to several pathologies, including diabetes and diabetes-related complications [33]. In this context, I aimed to improve the confocal analysis based on the dual-channel acquisition by using spectrally resolved fluorescence lifetime imaging of cells labeled with solvatochromic probes. This technique captures multiparametric information about the biophysical state

of the membranes, enabling a proficient distinction of membrane phases. Through this approach, each pixel is labeled by its intensity value, the solvatochromic spectral shift, and the spectrally-resolved fluorescence lifetime of the probe, thus providing information on the partition coefficient of the probe within the phase, the hydration level and the microviscosity of the phase, respectively. To perform a quantitative analysis, I introduced an artificial intelligence-based clustering tool (K-means) that, leveraging on the multiparametric content of spectrally resolved lifetime images, results in the extension of the spectrum of detectable membrane phases. This clustering method, which was already applied to fluorescence intensity and lifetime dataset [34,35], going beyond the classical two-state classification, provides the classification and the characterization of multiple phases and enables the real-time investigation with the sub-micrometric resolution of cell membrane phase transitions in living cells and tissues in different physiological and pathological conditions. I then applied this method to investigate the effects of membrane remodeling induced by high glucose levels on PC12 neuronal cells. High-glucose–induced neuronal cell death is indeed responsible for the development of diabetic complications [36], and although important abnormalities in energy metabolism have already been assessed [37], the related effects of membrane remodeling and lipid metabolism are still unclear.

Interestingly, although the separate analysis of the three maps, though indicating that some changes are occurring, does not provide the specificity needed to fully describe the phases of intracellular membranes, the proposed ML-based workflow [28] allowed evaluating the distribution of 8 different phases, thus characterizing the phase profiles of cells in different conditions, to investigate changes induced on the membrane phase state. Through this approach, we were thus able to study changes in membrane fluidity induced by different glucose concentrations in real-time.

4˙3. *From in-vitro models to clinical applications: membrane microfluidity of red blood cells*. – As high glucose levels are the main indicator, as well as clinical marker, of diabetes, we wondered whether and how factors influencing membrane fluidity could be monitored *in vivo* and how this *in vitro* observation could then be effectively translated to clinical practice. In this sense, we focused on the study of red blood cells (RBC), which reflect the body's more general systemic state and can therefore provide an important biomarker, using a minimally invasive analysis. RBC membrane fluidity, indeed, reflects a complex network of regulatory process that are affected by the systemic state. As an example, it has been shown that glycosylation-induced conformational changes of plasma membrane and cytosol proteins underline changes in the fluidity of lipid bilayer in diabetes. These changes impair several processes, as the glucose transport regulation by insulin, and contribute to the development of complications. Monitoring the modifications induced on the membrane physical state of the RBC can therefore furnish a sensitive index of disease progression, contextually allowing an indirect assessment of other cells membrane health status. To evaluate membrane microfluidity, we quantified the Generalized Polarization (GP) index, which ranges from -1 (highest membrane microfluidity) to $+1$ (lowest membrane microfluidity), allowing to distinguish the effects of the steady-state flux of phospholipids and cholesterol, as well as the environmentally-driven spatial phase reorganizations. Through this biomarker, we first investigated if RBC extracted from healthy and type 1 diabetic subjects (T1DM) are characterized by different values of microfluidity, since the systemic environmental parameters that alter proteins glycosylation and oxidation of proteins and membranes, as well as the dynamic flux of cholesterol between membranes and lipoproteins are different in healthy and diabetic subjects. The quantification of GP index revealed significant differences among

groups, with T1DM red blood cells associated with a decrease of GP index, thus reflecting the decrease in the extent of low microfluidity domains [38]. Since these results showed that erythrocyte membrane fluidity allows distinguishing between healthy and T1DM patients, we further focused on diabetic patients, to evaluate if changes in RBC fluidity occur within those developing diabetes-related microvascular complications and, in particular, diabetic retinopathy (DR) [39] and peripheral artery disease (PAD) [40]. Interestingly, a significant increase in membrane fluidity is retrieved in diabetic patients with confirmed diagnosis of DR or PAD with respect to diabetic subjects without any diabetes-related complications, considering comparable groups as per duration of the pathology. These results are consistent with those previously obtained by our group, according to which an increase in fluidity, a phase separation, and the formation of microdomains with different fluidity is retrieved in RBC membrane [41] of T1DM patients and T1DM patients with complications, as well as with time after the first diagnosis.

Although several mechanisms have been hypothesized to contribute to membrane fluidification observed in the disease, including systemic effects caused by chronic hyperglycemia, oxidative stress and metabolic alterations, the underlying metabolic problem is still open.

In this perspective, we further provided the functional and molecular characterization of another interesting system, constituted by human retinal pigment epithelium (ARPE-19) cells [42, 43] which, constituting the blood-retinal barrier, play an important role in preventing dangerous substances to enter the retina, as well as in maintaining a constant exchange of nutrients and metabolic useful molecules between the bloodstream and photoreceptors. In particular, to investigate potential therapeutic metabolic approaches, we evaluated the effect of the omega-3 polyunsaturated docosahexaenoic acid (DHA) on the oxidative stress and lipid metabolism alterations induced by high glucose levels on ARPE-19, highlighting that, through the modulation of the disruptive effects induced by hyperglycemia, it opens the possibility for a deeper investigation of metabolic approaches for the treatment of diabetes-related complications [44, 45].

5. – Conclusions

In conclusion, therefore, we have observed how metabolic imaging provides the tools to obtain information on various cellular mechanisms that may be involved in the onset of disease and its complications. However, while allowing for a great deal of information to be obtained, these tools require the alliance with artificial intelligence and machine learning approaches in order to investigate in depth metabolic properties otherwise not accessible. Resulting in a faster and more accurate analysis that is automatable and can provide decision-support tools, this approach paves the way for potential applications in clinical practice as well.

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