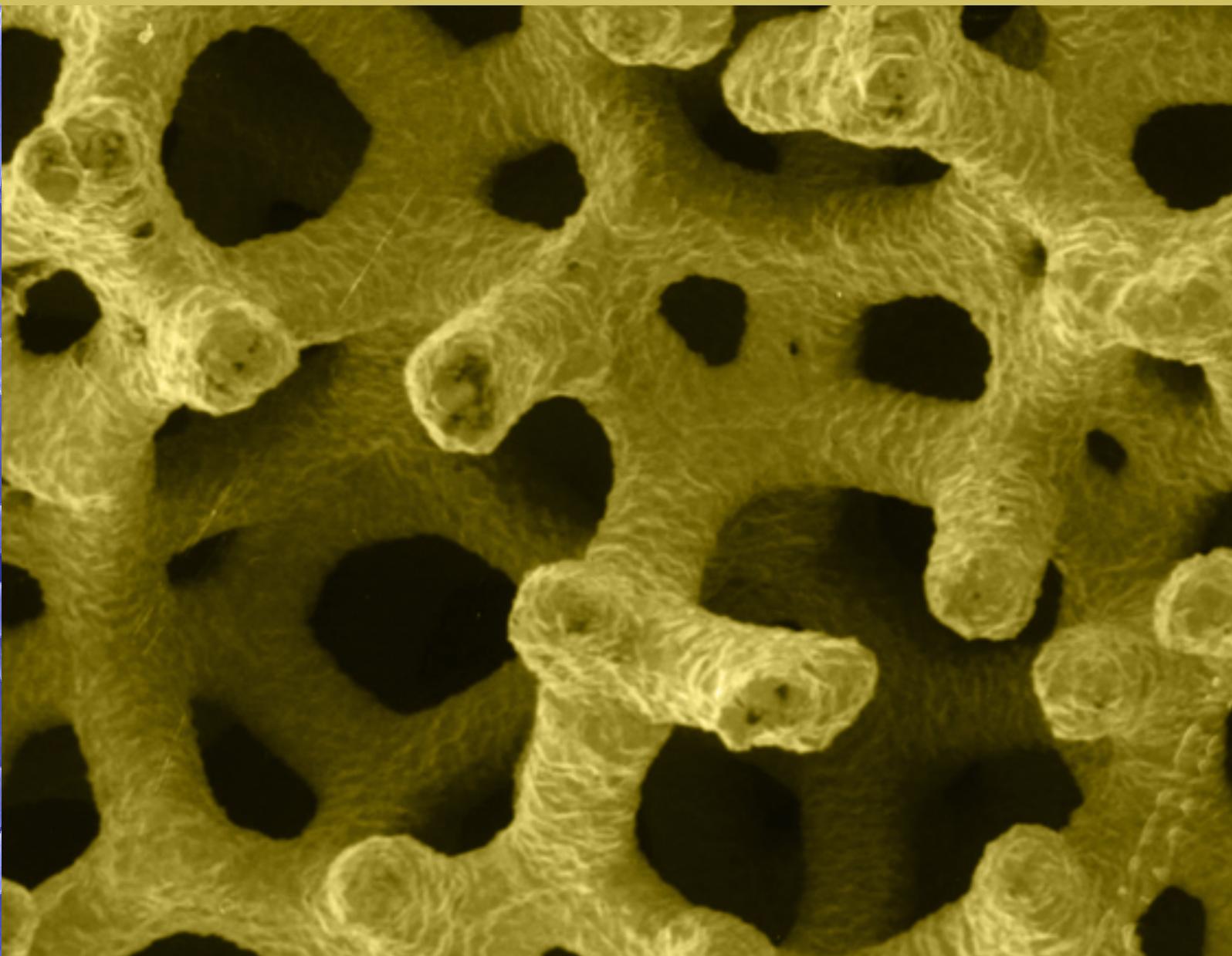


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Facultad de Ciencias Médicas; Universidad Nacional de La Plata;  
La Plata, Buenos Aires, Argentina. Tel.-Fax: +54-211-4834833  
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# THE CELL AS A GEL: MATERIALS FOR A CONCEPTUAL DISCUSSION

Luis A. Bagatolli<sup>1\*</sup> and Roberto P. Stock\*

<sup>1</sup> MEMPHYS-Center for Biomembrane Physics, University of Southern Denmark, Odense M, Denmark.

Campusvej 55, DK-5230 Odense Denmark

**\* Correspondence to:**

Luis Bagatolli (luisbagatolli@gmail.com)

Roberto Stock (rpstepoz@gmail.com)

## ABSTRACT

Recent results from our laboratory support the view that the intracellular milieu cannot be treated as a homogeneous dilute system and, more importantly, reveal for the first time a dynamical coupling between intracellular water and an active metabolic process involving fluctuations in ATP concentration. These results are difficult to understand in light of the premises that currently underpin the description of the function of cellular systems, e.g. van't Hoff's ideal solution theory, diffusion and mass action kinetics. Particularly, they emphasize the need to incorporate features of the cell interior that have been largely overlooked in the dominant model of the cell, such as crowding and limited availability of free water. This article discusses this problem by reconsidering an alternate view, called the association-induction hypothesis, which emphasizes the relevance of *emergent properties* of the cell cytosol during cellular function. This hypothesis provides a very reasonable theoretical framework to explain recently reported observations about the dynamical coupling of mechanochemical (i.e. viscoelastic) properties of the cell cytoplasm and cellular chemical transformations (metabolism).

## RESUMEN

Los resultados experimentales obtenidos recientemente en nuestro laboratorio apoyan la idea que el medio intracelular no puede ser tratado como un sistema homogéneo (o solución diluida), revelando además por primera vez un *acoplamiento dinámico entre el comportamiento colectivo del agua intracelular y un proceso metabólico activo que muestra fluctuaciones en la concentración de ATP*. Estos nuevos resultados -que son difíciles de interpretar en base a los supuestos más generalmente utilizados para interpretar las bases fisicoquímicas de la fisiología de los sistemas celulares (p.ej. teoría de las soluciones ideales de van't Hoff, difusión, y cinética de acción de masas)- subrayan la necesidad urgente de incorporar características importantes del interior celular, tales como el hacinamiento molecular y la escasa disponibilidad de agua libre. Este artículo analiza críticamente este problema considerando una hipótesis alternativa, llamada *hipótesis de asociación-inducción*, la cual hace hincapié en la importancia de las propiedades emergentes del citosol durante la función celular. Esta hipótesis proporciona un marco teórico razonable para explicar nuestras observaciones, particularmente el acoplamiento dinámico entre las propiedades mecanoquímicas (o viscoelásticas) del citoplasma celular y las transformaciones químicas (metabolismo) en el interior celular.

**Keywords:** Cell cytosol, emergent properties, hydrogels, oscillations in ATP concentration, Association-Induction Hypothesis. Citosol, propiedades emergentes, hidrogeles, oscilaciones en la concentración de ATP, Hipótesis de Asociación-Inducción.

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## Introduction

### *Brief historical overview of two distinct cellular models*

Right after the enunciation of the cell theory early in the 19<sup>th</sup> century two major ideas developed. One of them emphasized the uniqueness, in a physicochemical sense, of the entire cell substance, i.e. the cell cytosol. The second one focused its attention on a hypothetical thin surface, the plasma membrane. In the latter view –which became the dominant one– concepts such as osmotic pressure and membrane permeability to solutes were proposed to be closely related. Accordingly, intracellular water and solutes like  $K^+$  dissolved in the cell water are understood to exist in a physical state comparable to that of dilute solutions. This long tradition of visualizing living cells as fluid-filled vesicles, supported by the elegant solution theory of van't Hoff, provided the initial foundation for the *membrane theory*. Further evidence came from osmotic studies and from investigators in other specialized fields of cell physiology, such as J. Bernstein, author of the membrane theory of cellular electrical potentials (1902) and F. Donnan, author of the theory of membrane equilibrium of ionic distribution and electrical potential (1911). These theories and their corroborative evidence, which provided support for the membrane theory, follow from the fact that they share the same fundamental assumption: that living cells can be best conceived as membrane-enclosed dilute solutions. The interlinking theories mentioned above have also conjointly made the membrane theory the first coherent general theory of cell physiology. In other words, the membrane theory can satisfactorily integrated four major subjects of cell physiology: (i) cell volume control, (ii) selective solute distribution, (iii) selective solute permeability and (iv) cellular electrical potentials [1]. At present this conceptual framework dominates the outlook of cellular systems, although the view of the intracellular environment as a nanoscale replica of dilute systems is probably missing crucial information [2, 3]. It does not, for example, consider the peculiar physicochemical properties of the intracellular aqueous phase, treating it as a featureless isotropic environment in which chemical species move and react. A view that incorporates the altered colligative properties of the intracellular aqueous milieu would, however, provide a more comprehensive picture [2].

Back in 1861 Max Schultze, professor of Botany in Bonn, pronounced his protoplasmic doctrine, according to which a living cell is a membrane-less lump of protoplasm containing a nucleus. The view was supported by Thomas Huxley, who then stated that protoplasm constitutes the physical basis of life (for historical details see [1]). This view known as the *protoplasmic theory* evolved through the years developing a strong foundation in the physicochemical properties of colloidal systems, i.e. building on the observations that the behavior of most solutes inside cells does not usually resemble their behavior in dilute solutions. Cells are conceived in this model as colloidal systems that dynamically respond to fluctuations, either by dampening them or amplifying them cooperatively. Therefore, their properties are considered as *emergent properties* of organized supramolecular systems. Importantly and different from the membrane theory, which assumes intracellular water to exist in the liquid state, the cellular interior accommodates solutes based on adsorption sites and solubility properties of its colloidal water and is kept organized by central metabolism in a low entropy state which becomes responsive to environmental factors in very defined ways. Although important contributors to these ideas during the 20<sup>th</sup> century were Dmitrii Nasonov and Afanasy S.Troshin (among others), the most complete version of this perspective was provided in 1962 by Gilbert N. Ling, who called it the *Association-Induction (A-I) hypothesis* [4]. Ling's A-I hypothesis strongly challenges the modern mainstream consensus model of cellular membranes based on the fluid-mosaic model that envisions a lipid bilayer separating

the inside from the outside of cells with associated ion channels, pumps and transporters giving rise to the permeability processes of cells. A brief description of Ling's A-I hypothesis is provided below.

*Ling's Association-Induction hypothesis*

If water in the cell is comparable to dilute solutions, then all phenomena central to cell physiology, that is, integrated and coherent cellular behavior, will be the result of the properties of the membranes separating the various dilute aqueous compartments within the cell, and between the cell and the extracellular milieu. Three related phenomena that conform the core of cell physiology, namely, solute distributions, electric potentials and volume regulation, are today understood to map to the particular constitutions and activities of interfaces (membranes) between compartments. Marginal importance -if any-, is accorded to the bulk properties of the aqueous media on either side of the borders. In other words, intensive properties of the compartments are not considered to be of relevance as the activities that define the compartments lie at their boundaries; the composition and dynamics of compartments are the result of what their boundaries contain and, consequently, what they allow through or remove.

Gilbert Ling, following Troshin's Sorption Theory [5], developed a comprehensive statistical mechanical treatment of the behavior of solutes, both ionic and non-ionic, in the context of complex polyelectrolytes, as well as the response of the polymers themselves to the solutes. Both the theoretical developments and his careful experimentation were published in numerous articles and integrated in his barely known *A physical theory of the living state: The Association-Induction hypothesis* [4]. Although his conclusions are far reaching and difficult to summarize fairly, it would be accurate to state that he provides the first integrated description of the coupling between high molecular weight polymers (mostly proteins), small solutes (metabolites and ions) and the mechanical properties of the aqueous environment both at equilibrium (the 'resting state' of cells) and during cellular responses to stimuli (cellular action). Although Ling concentrated mostly on the physiology of muscle contraction and neural transmission, his theoretical framework extends well beyond and generalizes to all physiological phenomena. It provides also thorough formal interpretations of all three physiological phenomena mentioned above.

In the A-I Hypothesis, the influence of polymers and solutes on the integrated properties of the water-protein-metabolite system is a central feature of the structural and dynamic properties of cellular behavior. The products of central metabolic processes modulate the bulk properties of cells and cellular compartments, which in turn govern the interchange of ionic species ( $\text{Na}^+$ ,  $\text{K}^+$  and others) and metabolites based on differential adsorption and solubility in the crowded water phase. Specifically, Ling proposed that fluctuations in the activity of key metabolites (e.g. ATP) during an active metabolic process, through association with -and inductive effects on- alter the conformational states of fibrillar proteins (e.g. mostly those that participate in the cytoskeleton). This association-induction effect modulates the binding affinity of particular ions for proteins, altering also the structure of intracellular water (described by the polarized-oriented multilayer theory of cell water [6]) and, therefore, the partition coefficients of numerous molecular actors. In the particular case of ATP, Ling establishes that, upon interaction with the fibrillar protein matrix, it creates an *inductive effect* in the partially resonant polypeptide chains changing the electronic density of relevant chemical groups ( $\beta$ -, and  $\gamma$ -carboxyl groups, also the backbone NHCO groups), which in turn affect the affinity of protein for ions and water [6, 7].

In the current dominant view of the cell a semipermeable membrane divides two mostly aqueous phases (compartments) and, consequently, its key concepts for interpretation of cell structure and behavior are permeability and transport. Although the effect of molecular crowding has been somewhat acknowledged in updated versions of this model [8], water is still considered as a liquid solvent. In the A-I view, however, a more accurate model of the cell is the ion-exchange resin (i.e. the structured polyelectrolyte or fixed-charge system) and the tools for interpretation of physiological data are adsorption and partition coefficients [4, 9]. In more current terminology we could call the latter systems responsive hydrogels [10], capable of responding to fluctuations in their environments with many of the non-linear properties of living systems. In this respect some recent publications have addressed the fact that the cell cytosol can be treated as a poroelastic material [11], while a substantial body of work suggests that membrane-less organelles present in the cell cytosol can be treated as a multicomponent, viscous liquid-like structures that form via spontaneous phase transitions [12].

To finish this section it is interesting to examine what are the experimental evidence that motivated G. Ling to propose the A-I hypothesis as an alternative model. The author summarizes contentious results regarding important assumptions that sustain the membrane theory which can be briefly summarized in five main points:

- 1) Cell permeability to  $\text{Na}^+$  (see [13] and references therein)
- 2) Failure of Gibbs-Donnan equilibrium to predict ion distribution in cellular systems [14].
- 3) All or virtually all the water in the living cell assumes the dynamic structure of polarized-oriented multilayers [15].
- 4) Experimental data obtained in model systems -i.e. glass membranes, oil membranes, collodion (nitrocellulose) membrane, phospholipid bilayer membranes- points out that it is surface adsorption of ions rather than permeability that determines ion specificity of the measured electric potentials [7].
- 5) The minimum energy needed for the sodium pump is at least four times higher than, or 400% of the maximally available energy to the muscle cell, even if (1) the muscle spends all its energy on pumping sodium, and (2) all the essential energy conversion and utilization processes operate at 100% efficiency (see [13] and references therein).

## **2. ATP oscillations produced during oscillating glycolysis in yeast are coupled with the dynamics of intracellular water**

We recently provided evidence of a dynamical coupling between a metabolic (chemical) process and an emergent physical property of the cell cytoplasm (water relaxation) during oscillating glycolysis in yeast [16]. This study exploited the sensitivity of different 2-(acyl)-6-acylnaphtalene (DAN probes) derivatives introduced by Gregorio Weber in the late 70's, namely LAURDAN, PRODAN, ACDAN [17] (DAN probes) to monitor the dynamics of intracellular water during active metabolism, i.e. oscillating glycolysis. Weber proposed the use of these fluorescent reporters "as relaxation probes of various biological environments" [17]. The mechanism explaining the sensitivity of these probes to water dipolar relaxation<sup>1</sup>,

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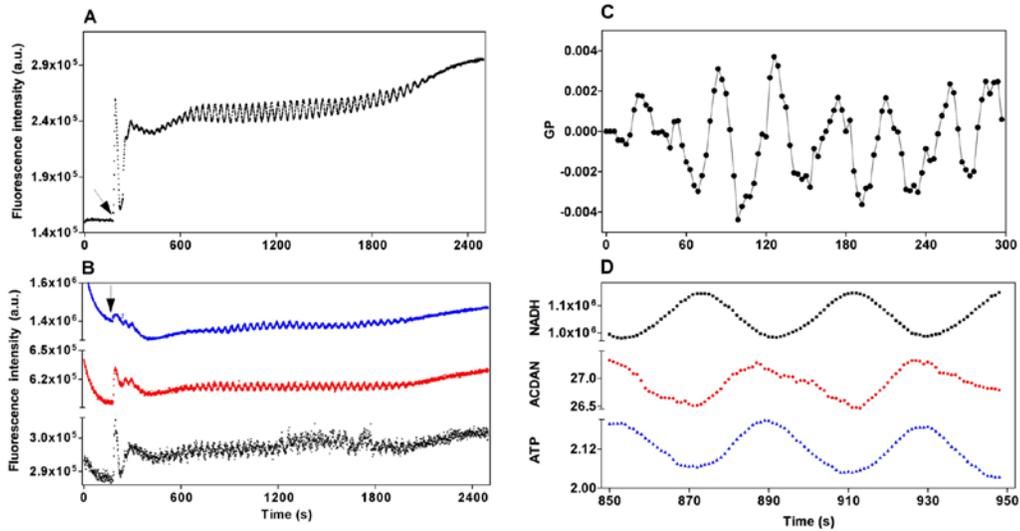
<sup>1</sup> we are not referring here to the well known effect of polarizability (also called general effects), e.g. observed in solvents with different dielectric constants and indexes of refraction (described by the Lippert model, [18]). This type of effect has been shown to be negligible to explain the sensitivity of these probes to, for example, different membrane packing in model and biological membranes [18].

which is a major player in the response of these probes to the dynamics of distinct aqueous environments, has been extensively reviewed [18]. Briefly, when the probe is in the excited state, prior to deactivation of its excess of energy via fluorescence, its excited state dipole (or transition moment) can specifically interact with its environment. If this environment has a strong dipolar character (e.g. water molecules) the dynamics of the surrounding dipoles will affect the energy of the probe's fluorescence transition. In other words, if the adjacent water molecules rotate (relax) on a similar time scale to the fluorescence lifetime of the probe, the energy of the probe's excited state will be diminished (by transfer to the surroundings) with respect to a reference state where no relaxation is present (e.g. water dipoles rotating slower than the probe's lifetime). The loss of energy of the excited state dipole by relaxation translates into a fluorescent emission of longer wavelength (it is shifted from blue to green in the DAN probes), reflecting a lower efficiency of excited state deactivation *by fluorescence* (or lower quantum yield). An extreme case happens in liquid water (which relaxes in the picosecond range) where the fluorescence emitted by the probe will be completely relaxed (520 nm, green color). In short, the DAN probes are exquisitely sensitive to water dynamics by virtue of its effect on both their spectral response and quantum yield.

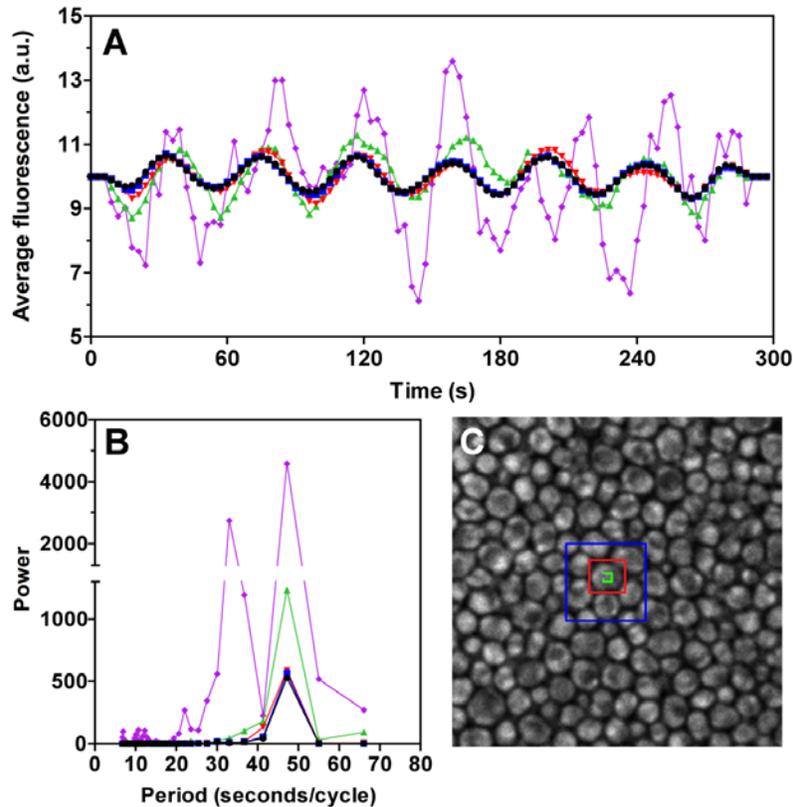
The oscillatory nature of glycolysis in *Saccharomyces cerevisiae* becomes apparent when unmasked by inhibition of respiration. As starved cells utilize glucose supplied in the medium, glycolysis products accumulate and disappear following a well-known waveform. Oscillations can be measured in real time following the intrinsic fluorescence of reduced nicotinic adenine dinucleotide, NADH [19, 20]. However, oscillations of other intracellular glycolytic intermediates [21], including ATP and intracellular pH [22] have been observed, suggesting the existence of underlying coupling mechanisms. Glycolytic oscillations are a property of single cells [20] but, at high cellular density, they become macroscopic since cells are quickly and robustly synchronized via diffusing metabolites. All models attempting to interpret these oscillations rely on the assumption that the intracellular milieu is a homogeneous environment where diffusing chemical species, consumed and produced by enzymes at particular rates, are responsible for the periodic accumulation and disappearance of measured metabolites.

#### *Experiments with DAN probes*

The most conspicuous observations arose when yeast exhibiting glycolytic oscillations were labeled with the DAN probes [16], both using cuvette fluorescence spectroscopy measurements and fluorescence microscopy. As shown in Figure 1 the fluorescence intensity of the probes fluctuates at the same frequency to that measured for NADH and ATP. This phenomenon, which is emission wavelength independent, is also reflected in oscillations measured in the Generalized Polarization (GP) function (Figure 1C, see reference [18] for details). Oscillations of the GP function in the cell yielding the measured changes in the intensity of emission (quantum yield) of the probes at any given wavelength can be explained only if solvent relaxation is the dominant mechanism. Importantly, spatially resolved information obtained from fluorescence microscopy experiments (Figure 2) demonstrate that oscillations of both NADH concentration and the DAN probes span all measured size scales, as established by measurements in progressively smaller regions of interest (ROIs), ranging from many cells to a single intracellular pixel (see Figure 2 for a representative example) [16].



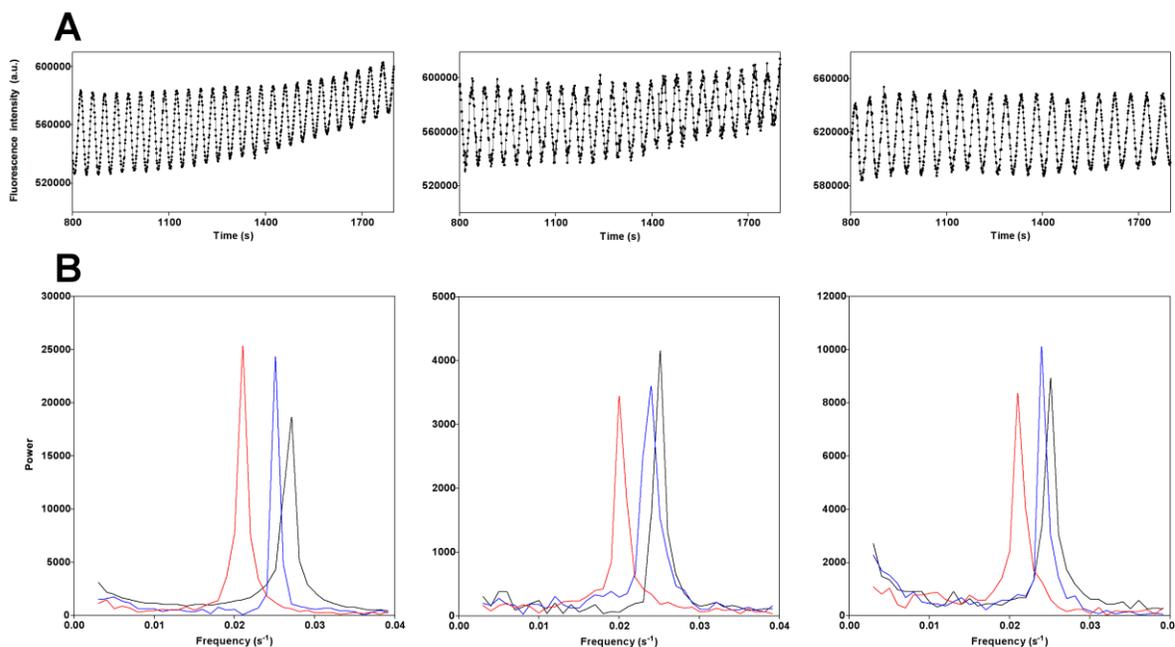
**Figure 1.** Oscillatory behavior of glycolysis and DAN probes in the fluorometer. Panel A) Oscillations of NADH. Panel B) Oscillations of ACDAN (red), PRODAN (blue) and LAURDAN (black). Panel C) Oscillations in the Generalized Polarization (GP) function (for ACDAN). Panel D) Phase relationships: ACDAN and NADH are expressed as fluorescence intensity, ATP is plotted in concentration units (mM). The arrows in panels A), B), and C) indicate the time of addition of 30 mM glucose followed by 5 mM KCN.



**Figure 2.** NADH oscillations in the microscope. Panel A) Running average of NADH oscillations at different scales. Black, whole image; Blue, 7 cells; Red, 1 cell; Green, 7x7 intracellular pixels; Purple, single pixel. Panel B) Power analysis of oscillations in each region. Panel C) Picture of NADH fluorescence ( $438 \pm 12$  nm) with color-coded regions of interest. Pixel size is  $0.1 \mu\text{m}$ , image corresponds to a field of  $25.6 \times 25.6 \mu\text{m}$ .

### Experiments with D<sub>2</sub>O

Pure water relaxes at an extremely fast rate [23], in picoseconds, yielding the observed ACDAN (or PRODAN) fluorescence emission maximum at 520 nm. Compared to water, D<sub>2</sub>O is denser, has higher freezing and boiling temperatures, and is more viscous. However, ACDAN and PRODAN in pure H<sub>2</sub>O and D<sub>2</sub>O display exactly the same emission peak (not shown, see [16]). In other words, *in bulk* (i.e. when the water or D<sub>2</sub>O dipoles reorient much faster than the excited state lifetime) the DAN probes are "blind" to any differences between normal and "heavy" water: both solvents draw the maximum possible energy from the excited state of the probes. However, when yeast cells are suspended in increasing concentrations of D<sub>2</sub>O (10%, 20% and 50% v/v) and the oscillations of NADH, ACDAN and PRODAN are measured, there is a significant decrease in their oscillation frequency (Figure 3A), which is apparent in the power spectrum representation (Figure 3B). It is important to notice that in resting cells, up to 50% D<sub>2</sub>O does not alter the emission spectrum of any of the DAN probes [16], consequently, the effect observed in Figure 3 is on the temporality of the oscillations, not on the probes. We propose that a more comprehensive explanation requires consideration of the well-known fact that the presence of deuterium affects the rates of reactions even if deuterated bonds are not themselves involved. This effect is termed the secondary isotope effect [24]; with deuterated water in the medium, it seems reasonable to conclude that the entire nanoenvironment where oscillating glycolysis occurs involves some degree of structure that is *dynamically* affected by the addition of a small amount of extra mass per molecule in the most abundant class of molecules [16].



**Figure 3.** The effect of D<sub>2</sub>O on NADH, ACDAN and PRODAN oscillations. (A) The top panels show NADH oscillations in the presence of no D<sub>2</sub>O, 10% D<sub>2</sub>O and 50% D<sub>2</sub>O (from left to right). (B) The bottom panels show the power spectra of the oscillations of NADH, ACDAN and PRODAN with increasing concentrations of D<sub>2</sub>O (black 0%, blue 10% and red 50%).

### 3. A challenge for the classical view describing the intracellular environment

It has been long known that the cellular environment is highly crowded with *very little water exhibiting the properties of dilute solutions* (for example, transverse relaxation times in muscle,[25]). Even in simple model systems, NMR studies of interfacial water indicate that it is quite dissimilar to dilute systems [26] and a recent perspectives article reflects upon the underestimated role of water in cell biology [27]. An explicit treatment of the dynamics of intracellular water should, therefore, provide elements for a more detailed structural, mechanistic and dynamical understanding of the coherence of cellular behavior, that is, the coupling between chemical and mechanical levels of description [28].

Our current framework of understanding of cellular processes relies on the premise that the cell cytosol is, at the relevant scale, like the dilute aqueous solutions in which we study biochemical processes *in vitro*. If this were true, water-soluble probes like ACDAN and PRODAN, sensitive to water dipolar relaxation dynamics, would not be expected to sense significant changes in the intracellular medium at this scale. In our view, the properties of the oscillations of the DAN probes are more consistent with the intracellular environment behaving as a responsive hydrogel, a view with very strong experimental support [10]. The study of hydrogels has traditionally relied on classical physicochemical measurements of equilibrium properties of the medium affected by crowding such as vapor pressure, swelling and shrinking. The results discussed in section 2 *provide robust direct spatial and temporal evidence of the intracellular aqueous phase as a medium exhibiting fast and coherent coupling of an intensive (scale invariant) cellular property (i.e. intracellular water relaxation) with a central metabolic process [16]*. Considering that polymerization/depolymerization of cytoskeletal structures is strongly dependent on ATP and ATPase activity [29-31], it is reasonable to suppose that ATP acts on the overall state of the cytoskeleton and that this impacts dipolar relaxation of the aqueous phase, possibly due to changes in viscoelastic properties. As metabolism oscillates so do interfacial water<sup>2</sup> dynamics; as D<sub>2</sub>O makes the system "heavier", all oscillations are synchronously slowed down. The chemistry and physics of the system are thus bidirectionally coupled. Solvent (water) motion has been shown to govern an important part of the energy landscape occupied by proteins, affecting catalysis [32, 33] and folding [34]. The observations reported in this review article provide a robust biological system for theoretical development and experimental testing of the idea that life depends on the maintenance of a low entropy state. The cytosol as a hydrogel, with most of its water dynamically coupled to central metabolic processes may provide the substrate where an entropic level of understanding of key processes of life can be found [35].

### 4. Concluding Remarks

Considering our results with the exquisitely sensitive DAN probes, we find ourselves in a situation where the dipolar relaxation phenomena as reported by the DAN fluorophores reveal coupling between (bio)chemical oscillations and some property of the major component of the cell (water). In the A-I hypothesis, ATP plays a critical role by keeping the cell at a low entropy state. The oscillations of water relaxation and their tight temporal coupling to the oscillating process (glycolysis) that cyclically produces and consumes ATP is a very

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<sup>2</sup> This term refers to polarized water molecules, that is, with restricted degrees of motion adjacent to the many interfaces in the cytosol. These motion restrictions result in different dynamical properties than those of "free" liquid water.

suggestive indicator of the power of the A-I interpretation of physiological processes. This coupling is difficult to explain in light of the canonical cell model, where water is considered a mostly passive liquid medium.

The A-I Hypothesis provides a rigorous body of theory and a wealth of experimental data to couple the chemical (metabolic transformations) to the physical (mechanical cellular responses), as well as tools for the interpretation of much of the most recent research on cellular physiology. Considering what we now know about polymer physical chemistry and excitability, and the tools we now possess to explore the behavior of living systems non-invasively and in multiple spatial and temporal scales, it may be high time to rethink the standard models that guide our thinking and interpretation of physiology using the A-I framework as a starting point.

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## About the authors



**Luis A. Bagatoli** obtained his MSc (1991) and PhD degree (1995) in Chemistry from the School of Chemical Sciences, University of Córdoba in Argentina. After a postdoctoral research at Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign, USA (1997-2000), and a brief stay in Argentina, he joined in 2002 MEMPHYS center at the University of Southern Denmark where he is currently a professor of biophysics. His main scientific interests are related to physicochemical aspects of biological systems, particularly membranous systems and the effects of molecular crowding in cells.



**Dr. Roberto P. Stock** is a Biochemist originally from Uruguay, now living in Mexico. Until 2014 he worked at the Instituto de Biotecnología of the Universidad Nacional Autónoma de México, where he led a research group on biochemistry, cell biology and animal venom toxicology.