The Use of 6-Acyl-2-(Dimethylamino) Naphthalenes as Relaxation Probes of Biological Environments

Luis A. Bagatolli and Roberto P. Stock

Abstract As Gregorio Weber anticipated in his seminal 1979 article, 6-acyl-2-(dimethylamino)naphthalene probes became excellent tools to study nanosecond relaxation processes of biological systems. Examples are the use of PRODAN (or DANCA) to study relaxation of specific protein matrixes, or LAURDAN (as well as PRODAN) extensively used to study the extent of water dipolar relaxation processes in biological membranes. In this chapter a novel application for this family of molecules is presented and discussed. Specifically, we show how these fluorescent probes can be used to monitor intracellular water dipolar relaxation in living cells displaying oscillatory metabolism. Our experimental results show a strong coupling between metabolism and intracellular water dynamics, challenging the view that water in the interior of cells exists mostly as a medium whose global properties are comparable to the properties of dilute solutions. The observed results can be very well interpreted in light of the association-induction hypothesis postulated by Gilbert Ling in 1962.

Keywords ATP • Fluorescent probes • Glycolysis • Molecular crowding • Oscillatory metabolism • Water dipolar relaxation

Contents

L.A. Bagatolli (✉) and R.P. Stock (✉)
Membrane Biophysics and Biophotonics Group/MEMPHYS – Center for Biomembrane Physics, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense, Denmark
e-mail: bagatolli@bmb.sdu.dk; rpstepoz@gmail.com

D.M. Jameson (ed.), *Perspectives on Fluorescence: A Tribute to Gregorio Weber*, Springer Ser Fluoresc (2016) 17: 197–216, DOI 10.1007/4243_2016_7,

Abbreviations

ACDAN	6-Acetyl-2-dimethylamine-naphthalene
DAN	2-(Dimethylamino)-6-acylnaphtalenes
GP	Generalized Polarization function
LAURDAN	6-Dodecanoyl-2-dimethylamine-naphthalene
PRODAN	6-Propionyl-2-dimethylamine-naphthalene

1 A Relevant Contribution from Gregorio Weber: The 2-(Dimethylamino)-6-Acylnaphtalenes Family

The displacement of the fluorescence spectrum to longer wavelengths (red shift) with increasing polarity of solvents has been the object of both theory and experiment since the 1950s. This type of red shift is largely dependent upon a large increase in the molecule's dipole moment in the fluorescent state over that of the ground state [1] and particular substituted aromatic molecules can satisfy this condition. Among his many seminal contributions to biological fluorescence in 1979 Gregorio Weber introduced a family of environmentally sensitive dyes based on a naphthalene structure substituted with electron donor (alkylamino group) and acceptor (acyl substituted carbonyl) groups in position 2 and 6, respectively [1]. This structure possesses a maximum distance between the electron donor and acceptor groups, resulting in a lowest excited state with an important charge transfer character. In his influential publication, Weber presented a general method for the synthesis of 2-(dimethylamino)-6-acylnaphtalenes, i.e., 6-propionyl (PRODAN), 6-acetyl (ACDAN), and 6-lauroyl (LAURDAN), together with a careful study of the response of PRODAN's absorption and fluorescence properties to solvents of different polarity. In this description the dipole interaction theory of Lippert [2] was considered and generalized to the other synthesized naphthalene derivatives [1]. For example, a 130 nm red shift (one of the largest reported to date) was described for PRODAN's emission maximum from cyclohexane to water with an important change in the magnitude of its transition dipole. Additionally, a significantly (larger) Stoke's shift was reported in solvents that can form hydrogen bonds (i.e., that can act as proton donors) with respect to aprotic solvents [1]. Later

on, further studies of PRODAN's response to solvent polarity were conducted in Weber's laboratory, where the Langevin distribution of electrostatic interactions was considered [3]. It is very important to remark that Weber's contribution went well beyond the rational design of environmentally sensitive fluorescent molecules and the characterization of their response in solvents with different polarity. An essential idea was the application of this knowledge to the study of biological material. More precisely, in 1979 Weber presented a study of the interaction of PRODAN with bovine serum albumin, proposing the use of this 2-(dimethylamino)-6-acylnaphtalene derivative as "*a relaxation probe of various biological environments*" [1].

In a later work, Weber exploited the nanosecond relaxation phenomenon in a protein matrix (addressed earlier in his laboratory [4]) to determine the polarity of the myoglobin haem pocket [5]. Specifically, Weber designed and used another 2,6 substituted naphthalene derivative, i.e., 6′-(*N*,*N*-dimethyl)amino-2-naphtophyl-4-trans-cyclohexanoic acid (DANCA) [5], a probe with greater affinity than PRODAN for apomyoglobin. In a similar way, Prendergast et al. synthesized 6-Acryloyl-2-dimethylaminonaphthalene (Acrylodan), a 6,2 substituted naphthalene derivative that selectively labels thiol moieties in proteins [6]. In the Prendergast et al. paper, the usefulness of Acrylodan in the study of "hydrophobic" domains, conformational changes, and dipolar relaxation processes in proteins was demonstrated by measurements of fluorescence spectra and lifetimes of a mercaptoethanol adduct in different solvents and of adducts of this agent with parvalbumin, troponin C, papain, and carbonic anhydrase [6].

In those early years the interest in environmental relaxation processes in biological systems was not restricted only to proteins. Similar ideas were applied as well to explore relaxation in membranes (i.e., model membranes but also biological membranes) using different 2-(dimethylamino)-6-acylnaphtalene derivatives introduced by Weber, such as LAURDAN, PRODAN, ACDAN [7–9], and 6-palmitoyl-2-[[(2-trimethylammonium)ethyl]methyl]amino] naphthalene (PATMAN), the last one introduced by Lakowicz et al. in 1983 [10, 11]. Other related derivatives had been also synthesized in Weber's group, i.e., 2-diisopropylamino-6-lauroylnaphthalene (LAURISAN), 2-methoxy-6-lauroylnapthalene (LAURMEN), and 2-hydroxy-6-lauroylnapthalene (LAURNA), and further characterized by Parasassi et al. [8]. Very recently, a new 2,6 naphthalene derivative has been introduced 6-dodecanoyl-2-[*N*-methyl-*N*-(carboxymethyl)amino]naphthalene (C-LAURDAN), to explore membranes mainly using fluorescence microscopy [12, 13].

The theme of this chapter is to discuss novel results recently obtained in our laboratory using some members of the 2-(dimethylamino)-6-acylnaphtalene family (called here DAN probes). Specifically, we discuss a new strategy that uses ACDAN, PRODAN, and LAURDAN (Fig. 1) to monitor the dynamics of intracellular water in cells displaying metabolic oscillations [14]. Because of their different partition properties and shared response to solvent dipolar relaxation, these DAN probes were chosen as sensors to monitor heterogeneities in the collective dynamics of the most abundant intracellular dipoles, namely water molecules. In this work we

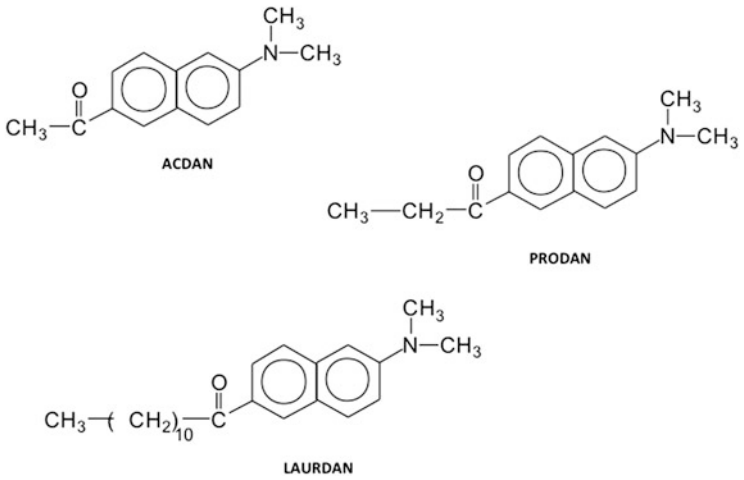

Fig. 1 Chemical structures of different 2,6 substituted naphthalene derivatives

took advantage of the oscillatory behavior of glycolysis in yeast cells to add a temporal dimension (phase and period) that would allow us to establish correlations between the fluorescence dynamics of the probes and metabolism. Before discussing this new material a brief review of important features of these probes will be presented in the two following sections.

2 A Model for DAN Relaxation in Biological Environments

A model to explain the fluorescence response of DAN probes in biological environments was proposed for LAURDAN and PRODAN inserted in lipid bilayers. Once these probes are incorporated in glycerophospholipid membranes an emission red shift of ~50 nm is observed when the membrane undergoes a solid-ordered (s_o) to liquid-disordered (l_d) phase transition [15–17]. Since changes in the "static" dielectric constant between the two membrane phase states are not sufficient to explain the observed fluorescence emission shift, a model to interpret these changes was originally provided by Parasassi et al. [17]. These authors anticipated that the nanosecond relaxation process observed in the l_d phase [15, 16, 18] is caused by the presence of water molecules with restricted mobility in the region where the probe is located (near the glycerol backbone of the glycerophospholipids) [17, 19, 20]. Part of probe's excited state energy is utilized for the reorientation of the water dipoles diminishing the singlet excited state (S_1) energy (see Fig. 2) and so shifting the emission spectrum of the probe to longer wavelengths. Notice that the emission shift is accompanied by a decrease in the lifetime of the probe and, consequently, in its quantum yield. Importantly, the relaxation caused by these water molecules is different to the water molecules existing in bulk phase that have

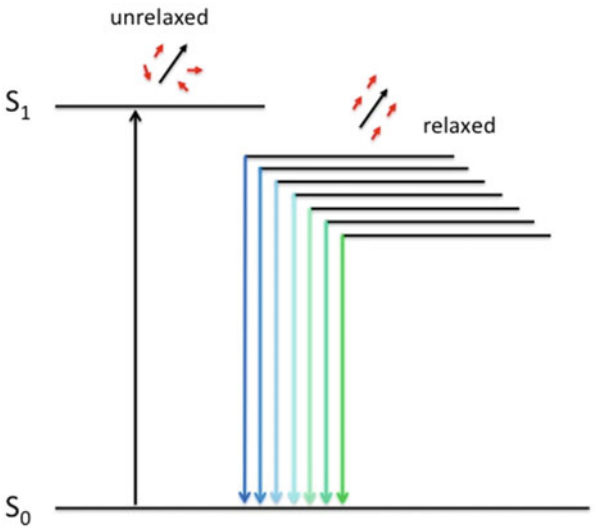

Fig. 2 Schematics of the ground (S_0) and excited state (S_1) energy levels in the presence of the solvent dipolar relaxation. S_1 decreases in energy as solvent dipolar relaxation proceeds

an orientational relaxation time below one picosecond [17]. Relaxation times have been measured for the l_d and s_o phases using an expression equivalent to the classical Perrin equation for the generalized polarization function (GP, for details see below, Sect. 3), assuming a two-state process [18]. These values were reported to be 2.5×10^9 s^{-1} and 4×10^7 s^{-1} for the l_d and s_o phases, respectively.

There is considerable experimental evidence that supports the idea that the particular dynamics of structured water molecules in the vicinity of the probe are the cause of the observed emission shift in membranes undergoing a phase transition [8, 21]. For example, experiments in membranes composed of DMPC in D_2O show slower relaxation dynamics at and above the phase transition, although the main phase transition temperature is the same with respect to that observed for the same lipid membranes in H_2O [8]. This important observation, which cannot be the consequence of a chemical reaction because the transition involves no compositional changes, points that the greater mass of the ensemble of (membrane-associated) D_2O molecules reorienting (sensed by LAURDAN) as the supramolecular structure changes is enough to slow down the transition.

A very useful parameter to monitor transitions between these unrelaxed and relaxed states was introduced by Enrico Gratton and his group of collaborators in 1990 [16]. This parameter, called the generalized polarization, has become very popular to study membrane and will be briefly discussed in the next section.

3 The Generalized Polarization Function

The generalized polarization (GP) function was defined to exploit a simple steady state parameter (the probe's emission spectra) to study structural and dynamical processes in model and biological membranes. Specifically, the GP function was introduced to quantitatively determine the relative amounts and temporal fluctuations of two distinct lipid phases when they coexist in a model membrane (for comprehensive reviews see [7, 8, 21]). This function was originally defined as

$$\mathrm{GP} = \frac{I_\mathrm{B} - I_\mathrm{R}}{I_\mathrm{B} + I_\mathrm{R}} \tag{1}$$

where I_B and I_R are the measured fluorescence intensities under conditions in which wavelength (or a band of wavelengths) B (for blue shifted) and R (for red shifted) are both observed using a given excitation wavelength. Being a weighted difference, the values of the GP must fall within -1 and 1; the lower this value the greater the extent of bathochromic shift in the fluorescence emission spectrum. This definition is formally identical to the classical definition of fluorescence polarization, in which B and R represent two orthogonal orientations of the observation polarizers in the fluorometer. The advantage of the GP function for the analysis of the spectral properties of the DAN probes is derived from the well-known properties of the classical polarization function, which contains information on the interconversion between two different "states" of the emitting dipole of the fluorophore. In the original studies, the LAURDAN GP was shown to distinguish between the extent of water relaxation in a phospholipid membrane, which is very low in a solid-ordered (gel) phase with respect to a liquid-disordered phase [7, 8, 21]; importantly, it was possible to distinguish fluctuations in the GP values *only* when the two lipid phases coexist. In general terms the states B and R will, respectively, correspond to unrelaxed and relaxed environments sensed by the probes. In the particular case of yeast cells displaying oscillating glycolysis that will be addressed in the remaining sections of this chapter, a generalization of the use of the GP function is applied, i.e., we explore fluctuations in water relaxation throughout the cell rather than in just membrane-associated water.

4 Glycolytic Oscillation in Yeast and the State of Intracellular Water in the Cell

The oscillatory behavior of many biological processes has been studied for decades. Examples include slow genetic oscillations of circadian rhythms [22], periodic pattern formation in embryogenesis [23], oscillating cytoskeletal structure in mechano-sensitive hair bundles in the auditory system, and, at the single cell level, the oscillations of Min gene products in *Escherichia coli* that dynamically

determine the site of cell division, among others [24]. The oscillatory nature of glycolysis in *Saccharomyces cerevisiae* becomes apparent when unmasked by inhibition of respiration. As 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 nicotine adenine dinucleotide, NADH [25, 26]. However, oscillations of other intracellular glycolytic intermediates [27], CO_2 [28], mitochondrial potential, ATP, and intracellular pH [29] have been observed, suggesting the existence of underlying coupling mechanisms. Glycolytic oscillations are a property of single cells [26] but, at high cellular density, they become macroscopic since cells are quickly and robustly synchronized via diffusing metabolites. As described previously in the literature, these oscillations are induced by synchronizing the cells by starvation, followed by addition of cyanide (or argon), which inhibits the respiratory chain, and glucose (which provides the substrate for continued glycolysis); for details see [14].

Attempts to understand oscillating glycolysis have generally taken the form of models of a few to tens of enzymatic reactions and some rate-controlling steps (e.g., phosphofructokinase). However, a more recent and kinetically equivalent proposal of control of oscillations by sugar transport has established that the origin of oscillations is diffuse and not governed by a single reaction, whether chemical transformation or sugar transport [30]. All models have in common that they are premised on mass action kinetics. In other words, they rely on the assumption that the intracellular milieu is, at a relevant scale, a homogeneous environment where diffusing chemical species, consumed and produced by enzymes at particular rates (with requisite delays, [31]), are responsible for the periodic accumulation and disappearance of measured metabolites.

This view of the intracellular environment as a nanoscale replica of dilute systems, however, is probably missing crucial information [32, 33]. It does not consider the peculiar physicochemical properties of the intracellular aqueous phase, treating it as a featureless isotropic environment in which chemical species move and react. This view has an honorable history going back to van't Hoff's insight that solutes could, in dilute conditions, be treated with formalisms successfully applied to gases. A view that incorporates the altered colligative properties of the intracellular aqueous milieu would, however, provide a more comprehensive picture [32]. Considering what is now known of the behavior of hydrogel materials [34], coherent macroscopic behavior in cells or sets of cells (physiology) can be conceived as the result of dynamical coupling of mechanochemical (i.e., viscoelastic) properties of the medium to chemical transformations (metabolism).

Water constitutes the most abundant component of the cell. Its unusual properties as a polar solvent have been recognized as part and parcel of life processes [35]. While van't Hoff's insight has proven invaluable, it reaches its limit of usefulness when water itself is strongly impacted by solutes (that is, deviates from tractable ideality). Furthermore, water in the cell is not just a medium where reactions occur but an active participant, e.g., many cellular reactions are condensations that produce water, or hydrolytic ones, that consume it. 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, [36]). Even in simple model systems, NMR studies of interfacial water indicate that its properties are quite dissimilar to dilute systems (see [37]). Many cellular processes, such as secretion and endocytosis, have been productively modeled by responsive hydrogels [38]. 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 [39]. For such a treatment to be feasible, however, time resolved information on intracellular water behavior is essential.

5 Response of the DAN Probes in Resting and Oscillating Yeast Cells

The idea of transitions between unrelaxed and relaxed environments, which are included in the model presented in Sect. 2 for membranes, can be extended to other systems such as the cell cytoplasm. Thus, we extended the notion to use ACDAN, PRODAN, and LAURDAN to study cell-wide intracellular water dynamics [14]. These DAN probes constitute a series in which the fluorescent group is coupled to progressively longer hydrophobic chains (see Fig. 1), thus exhibiting different affinities for hydrophobic/hydrophilic environments [9]. The shortest of these (C_2, ACDAN) is essentially insensitive to membrane-associated water dynamics since it partitions to hydrophilic environments. The next (C_3, PRODAN) partitions to both membranes and surrounding water. The most hydrophobic probe contains a lauroyl chain (C_{12}, LAURDAN), which in principle places it squarely within bilayers as its solubility in water is negligible. Thus the partition properties of these different molecules allow the exploration of a broad range of intracellular environments, which is important to establish whether the measured responses constitute a global cellular phenomenon [14]. Results observed in resting cells, which relate the spatial distribution of the probe with their fluorescence emission response, demonstrated a heterogeneous environment in terms of the extent of intracellular water dipolar relaxation across the cell cytoplasm. Interestingly, the three probes show two discernible populations with maxima in the green (G, $\approx$490 nm) and blue (B, $\approx$440 nm). An example is depicted in Fig. 3 for ACDAN (observed both in the fluorometer and the fluorescence microscope) where the fluorescence intensity ratio between these two bands (G/B) decreases in the order ACDAN>PRODAN>LAURDAN following the affinity of the probes for hydrophilic environments (ACDAN is the most water soluble). In any case, the spatial distribution of LAURDAN is not far from that observed with the other two probes, although its overall fluorescence emission band is narrower [14]. Moreover and very important, the fluorescence emission spectra of the DAN probes are far from

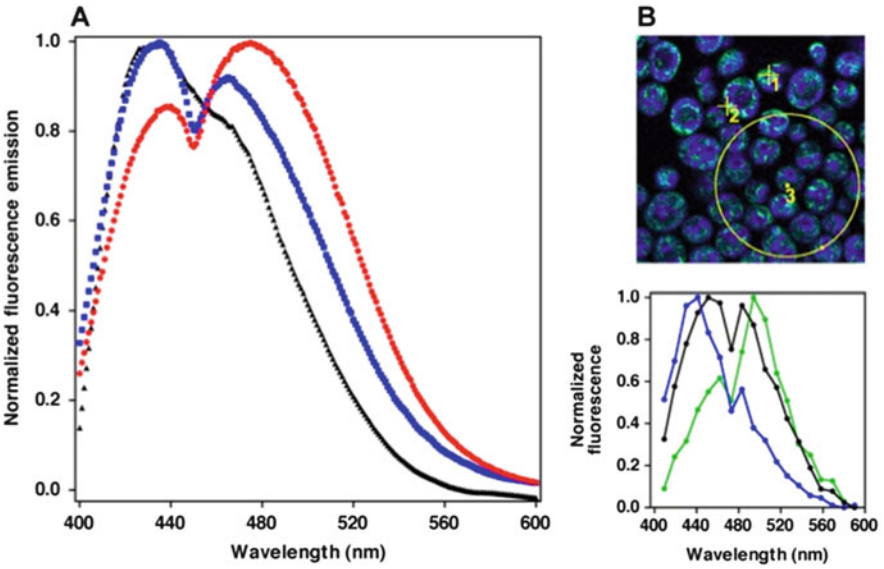

Fig. 3 Fluorescence response of DAN probes in resting cells. Panel (**a**) Emission spectra of cells labeled with ACDAN (*red*), PRODAN (*blue*), and LAURDAN (*black*) measured in the fluorometer. Panel (**b**) Spectral image of cells labeled with ACDAN (*top*) with spectra (*bottom*) of selected regions of interest: single B region (*blue*, ROI 1), single G region (*green*, ROI 2), and the overall spectrum (*black*, large circle defines ROI 3). Spectral resolution in the microscope is lower than in the spectrofluorometer. Image size is 15×15 μm. The spectral images of PRODAN and LAURDAN are not shown (the reader can find this information in Ref. [14])

what is expected from liquid water (where the emission maximum is located around 520 nm). In light of these results the application of the model discussed in Sect. 2 is justified by the observation that the DAN probes detect two main fluorescence contributions, which we assigned to correspond to unrelaxed and relaxed states of the probe, Fig. 3.

The most conspicuous observation arose when yeast labeled with the DAN probes exhibits glycolytic oscillations. This has been observed both using cuvette fluorescence spectroscopy measurements and fluorescence microscopy [14]. As shown in Fig. 4 the fluorescence intensity of these reporters 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 GP function (Fig. 4c). Following the model presented in Sect. 2, 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 show that oscillations of both NADH concentration and the DAN probes span all measured size scales, as established by multiphoton excitation microscopy in progressively

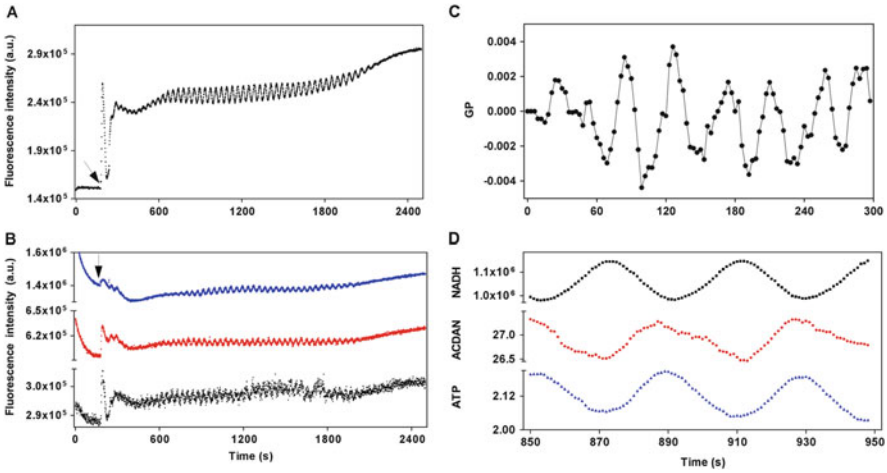

Fig. 4 Oscillatory behavior of glycolysis and DAN probes in the fluorometer. Panel (**a**) Oscillations of NADH. Panel (**b**) Oscillations of ACDAN (*red*), PRODAN (*blue*), and LAURDAN (*black*). Panel (**c**) Oscillations in the Generalized Polarization (GP) function (for ACDAN). Panel (**d**) Phase 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

smaller regions of interest (ROIs), ranging from many cells to a single intracellular pixel (see Fig. 5 for a representative example) [14].

Control experiments were performed to confirm whether the oscillations observed for DAN probes have a different origin to those included in the above-mentioned model. For example, similar experiments using ANS – which specifically interacts with membranes and proteins and shows negligible fluorescence emission when dissolved in liquid water [40] – show a total lack of oscillatory behavior, arguing against oscillations originating in interactions not mediated by water [14]. Additionally, iodoacetate (a blocker of glycolysis) causes a correlated disappearance of all oscillations (NADH, ATP, and DAN probes) and, as ATP is depleted, a bathochromic spectral response of the probes is observed. *All these observations indicate that water properties are coupled with the metabolic process that generates ATP in the intracellular environment*. It is important to note that ATP/ADP by themselves in a crowded solution do not affect ACDAN fluorescence [14], suggesting that it is the cellular environment that is responsible for the oscillatory behavior of the probe.

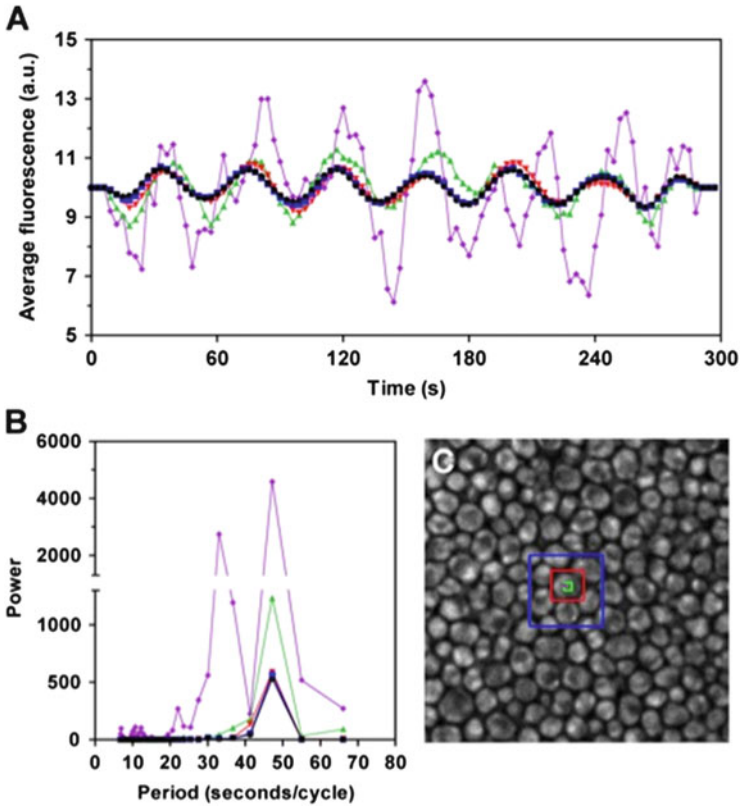

Fig. 5 NADH oscillations in the microscope. Panel (**a**) Running average of NADH oscillations at different scales. *Black*, whole image; *Blue*, 7 cells; *Red*, 1 cell; *Green*, 7×7 intracellular pixels; and *Purple*, single pixel. Panel (**b**) Power analysis of oscillations in each region. Panel (**c**) Picture of NADH fluorescence (438 ± 12 nm) with color-coded regions of interest. Pixel size is 0.1 μm, image corresponds to a field of 25.6×25.6 μm

6 Experiments with D_2O

Pure water relaxes at an extremely fast rate [41], in picoseconds, yielding the observed ACDAN (or PRODAN) fluorescence emission maximum at 520 nm. Compared to water, D_2O is denser, has higher freezing and boiling temperatures, and is more viscous. However, ACDAN and PRODAN in pure H_2O and D_2O display exactly the same emission peak (Fig. 6). In other words, *in bulk* (i.e., when 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_2O (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 (Fig. 7a), which is

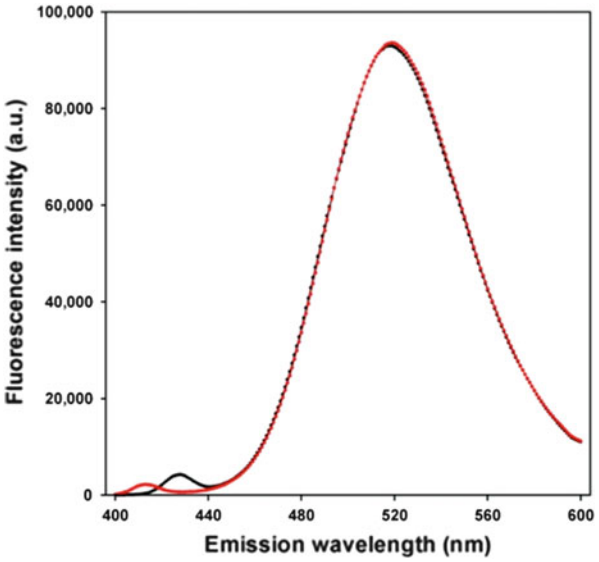

Fig. 6 Fluorescence of ACDAN in solvents. Raw fluorescence emission spectra of 5 μM ACDAN in pure H_2O (*black*) and pure D_2O (*red*). The lower peak at shorter wavelengths corresponds to the Raman effect of the solvent. The same behavior was observed for PRODAN

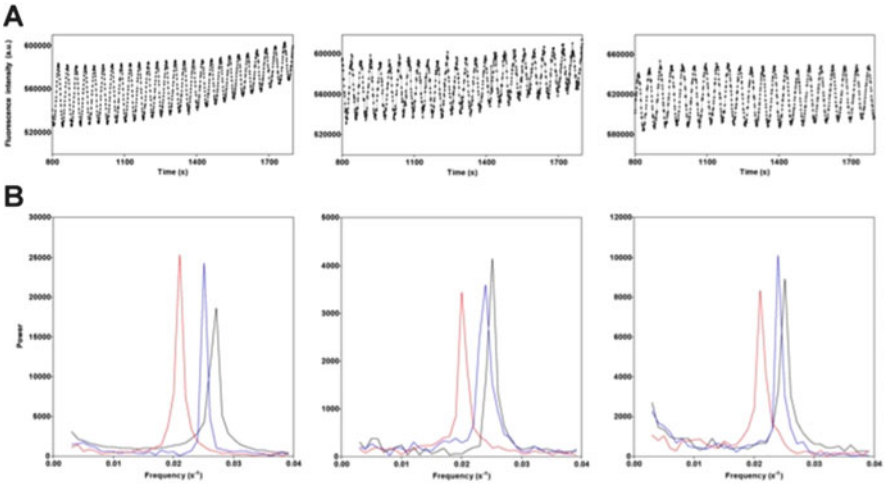

Fig. 7 The effect of D_2O on NADH, ACDAN, and PRODAN oscillations. (**a**) The *top panels* show NADH oscillations in the presence of no D_2O, 10% D_2O, and 50% D_2O (from *left* to *right*). (**b**) The *bottom panels* show the power spectra of the oscillations of NADH, ACDAN, and PRODAN with increasing concentrations of D_2O (*black* 0%, *blue* 10%, and *red* 50%)

apparent in the power spectrum representation (Fig. 7b). It is important to notice that in resting cells, 50% D_2O does not alter the emission spectrum of any of the DAN probes [14], consequently, the effect observed in Fig. 7 is on the temporality of the oscillations, not on the probes.

Heavy water has many biological effects [42]. Its impact on cytoskeletal dynamics is well documented although quantitative explanations are still under investigation [43]. The lower frequency of glycolytic oscillations in the presence of D_2O might be conceived as a consequence of the fact that deuterated compounds are chemically more stable and that the rates of reactions involving deuterated bonds can be slower. However, while this would be an apparently straightforward explanation for the *chemical transformations* very much in the spirit of van't Hoff, it fails to explain why the oscillations of the DAN probes occur at all, and why they remain synchronous with the slowed down ATP oscillations. It is important to stress that it is the *frequency* of all oscillations that is affected by D_2O, not the response of the probes [14].

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 (cf. membrane phase transitions mentioned in Sect. 2). This effect is termed the secondary isotope effect [42]; 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 [14]. These results also sustain the exquisite sensitivity of these probes to detect changes due to solvent dipolar relaxation in biological systems.

7 A Challenge for the Classical View Describing the Intracellular Environment

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, partially water-soluble probes like ACDAN and PRODAN, sensitive to 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 [34]. 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 presented along this chapter *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* [14]. This conclusion challenges the use of the concept of "normal" diffusion, a corollary of van't Hoff's theory of ideal solutions, to explain and build models of integrated cellular behavior. The coupling, at multiple scales, of water dynamics to ATP levels during glycolytic oscillations may offer a more complete perspective of the category of phenomena generally described as anomalous diffusion. In fact, the very term "anomalous diffusion" is an acknowledgement that the environment inhabited by intracellular molecules of interest – in this case a key product of glycolysis whose concentration oscillates – cannot be accurately described in terms of concepts derived from the chemistry of dilute solutions, or diffusion in terms of a straightforward application of the Stokes–Einstein relationship.

Considering that polymerization/depolymerization of cytoskeletal structures is strongly dependent on ATP and ATPase activity [43–45], 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 dynamics; as D_2O 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 [46, 47] and folding [48]. The observations reported in this chapter provide a robust biological system for theoretical development and experimental testing of Erwin Schrodinger's insight that life depends on the maintenance of a low entropy state [49, 50]. 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 [51].

But is there any useful model that can help us to explain this phenomenon?

8 Coupling Between Intracellular Water Dynamics and Metabolism: A Call for Models

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, the once opponent of the concept of protoplasm, who then stated that protoplasm constitutes the physical basis of life. Without going into excessive historical detail, this view known as protoplasmatic theory evolved along 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 necessarily resemble their behavior in dilute solutions. In this model cells are conceived as colloidal systems that dynamically respond to fluctuations, either by dampening them or by 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 it 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 twentieth century were Dmitrii Nasonov and Afanasy S. Troshin (among others), the most complete version of this theory was provided in 1962 by Gilbert N. Ling, who called it the *Association-Induction* (*A-I*) *hypothesis* [49]. 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. In addition, Ling's A-I hypothesis is not giving any relevant role to lipids or to their supramolecular structures, emphasizing that interactions among proteins, water, salts, and relevant metabolites are the dominant parameters for cellular functioning. A brief description is provided below. For detailed (and updated) sources describing these concepts see references [52, 53]. In particular, reference [52] introduces an interesting idea to explain the macroscopic physical properties of the cell cytoplasm, i.e., a basic structural unit composed of proteins, water, ATP, and ions he termed the nano-protoplasm.

8.1 The 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 (i.e., scale-invariant) properties of the compartments are not considered to be of relevance as the activities that define the compartments lie at their boundaries; their compositions and dynamics are the result of what their boundaries contain and, consequently, what they allow through or remove.

Gilbert Ling, following Troshin's Sorption Theory [54], 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 monumental, and barely known, *A physical theory of the living state*: *The*

Association-Induction hypothesis [49]. Although his conclusions are far reaching and difficult to summarize fairly, it would be fair 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 [52, 53]. It provides thorough interpretations of all three physiological phenomena mentioned above, and a context for the observations we report in our study of metabolic oscillations and water dipolar relaxation.

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 (Na^+, 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 metabolites (e.g., ATP) during an active metabolic process, through association with – an inductive effect on – alter the conformational states of fibrillar proteins (e.g., those that participate in the cytoskeleton). This association-induction effect modulates the binding affinity of these ions for proteins, changing also the structure of intracellular water (described by the polarized-oriented multilayer theory of cell water [52]) and, therefore, the partition coefficients of numerous molecular actors. In the current dominant view of the cell the conceptual model is the semipermeable membrane that divides two mostly aqueous phases (compartments) and, consequently, its key concepts for interpretation of cell structure and behavior are permeability and transport. In the A-I view, however, a more accurate model 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 [49, 53]. In more current terminology we could call the latter systems responsive hydrogels, capable of responding to fluctuations in their environments with many of the non-linear properties of living systems.

9 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 aqueous component of the cell. 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 are a very 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 passive liquid medium.

As an author put it, "It needs, perhaps, a certain intellectual effort to accept that (...) highly specific and regularly localised events (...) can be generated by interactions of vaguely spread, almost or completely constant parameters with some quite degenerate, occasionally localised dynamical perturbations, rather than by strictly specific and precisely located events" [55].

The A-I hypothesis provides a rigorous body of theory and a wealth of experimental data to couple the chemical (metabolic transformations) and physical (mechanical cellular responses), as well as tools for the interpretation of much of the most recent research on cellular physiology, unavailable at the time of Schrödinger and Ling. 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.

A Note from the First Author

The first author of this paper was extremely privileged to meet Gregorio Weber for first time during his PhD studies in Argentina, specifically at the meeting of the Argentinian Biophysical Society that took place in the beautiful hills of Villa Giardino, province of Córdoba in November of 1994:

> My PhD advisor requested help to pick up Gregorio Weber at a Hotel in the city of Córdoba and drive him to Villa Giardino. I obviously pushed very hard to (successfully) get that duty; being a young student it was a moment of great excitement to have the chance to meet "the professor". I will never forget the enthusiasm and expectations I had. Biological fluorescence was one of my favorite topics, which largely influenced on my decision to pursue a PhD in biophysics at the University of Córdoba in Argentina. I had everything planned, I had at least 1 h of travel from Córdoba to Villa Giardino, so I prepared many questions based on my study of his seminal papers on fluorescence. My plan however was not accomplished at all and I ended up hearing an amazing history of Gregorio as a young boy, travelling in a train through the Córdoba hills for vacations. With his wording he had the ability to transform the beautiful landscape around us into a 1920s postcard scene. Notwithstanding a fruitful discussion in my poster, during that meeting he also gave me advice that influenced enormously my scientific career: to join Enrico Gratton's group as a post-doc. Another relevant lesson from the professor came later in that meeting, when he gave an hour-long closing talk on fluorescence using only one overhead! Needless to say, one of the most interesting and inspiring talks I have ever attended in my entire professional career.

Acknowledgements This work is supported by a grant from the Danish Research Council (FNU, 12-124751 0602-02507B).

References

1. Weber G, Farris FJ (1979) Synthesis and spectral properties of a hydrophobic fluorescent probe: 6-propionyl-2-(dimethylamino)naphthalene. Biochemistry 18(14):3075–3078
2. Lippert E (1957) Spektroskopische Bestimmung des Dipolmomentes aromatischer Verbindungen im ersten angeregten Singulettzustand. Z Elektrochem 61:962–975
3. Macgregor RB, Weber G (1981) Fluorophores in polar media: spectral effects of the Langevin distribution of electrostatic interactions. Ann NY Acad Sci 366:140–154
4. Lakowicz JR, Weber G (1973) Quenching of protein fluorescence by oxygen. Detection of structural fluctuations in proteins on the nanosecond time scale. Biochemistry 12 (21):4171–4179
5. Macgregor RB, Weber G (1986) Estimation of the polarity of the protein interior by optical spectroscopy. Nature 319(6048):70–73
6. Prendergast FG, Meyer M, Carlson GL, Iida S, Potter JD (1983) Synthesis, spectral properties, and use of 6-acryloyl-2-dimethylaminonaphthalene (Acrylodan). A thiol-selective, polarity-sensitive fluorescent probe. J Biol Chem 258(12):7541–7544
7. Parasassi T, Gratton E (1995) Membrane lipid domains and dynamics as detected by LAURDAN fluorescence. J Fluoresc 5(1):59–69
8. Parasassi T, Krasnowska EK, Bagatolli L, Gratton E (1998) LAURDAN and PRODAN as polarity-sensitive fluorescent membrane probes. J Fluoresc 8(4):365–373
9. Zeng J, Chong PL (1995) Effect of ethanol-induced lipid interdigitation on the membrane solubility of Prodan, Acdan, and Laurdan. Biophys J 68(2):567–573
10. Lakowicz JR, Bevan DR, Maliwal BP, Cherek H, Balter A (1983) Synthesis and characterization of a fluorescence probe of the phase transition and dynamic properties of membranes. Biochemistry 22:5714–5722
11. Jurkiewicz P, Sykora J, Olzynska A, Humpolickova J, Hof M (2005) Solvent relaxation in phospholipid bilayers: principles and recent applications. J Fluoresc 15(6):883–894
12. Kim HM et al (2007) A two-photon fluorescent probe for lipid raft imaging: C-laurdan. Chembiochem 8(5):553–559
13. Dodes Traian MM, Gonzalez Flecha L, Levi V (2012) Imaging lipid lateral organization in membranes with C-laurdan in a confocal microscope. J Lipid Res 53(3):609–616
14. Thoke HS et al (2015) Tight coupling of metabolic oscillations and intracellular water dynamics in saccharomyces cerevisiae. PLoS One 10(2), e0117308
15. Parasassi T, Conti F, Gratton E (1986) Time-resolved fluorescence emission spectra of laurdan in phospholipid vesicles by multifrequency phase and modulation fluorometry. Cell Mol Biol 32(1):103–108
16. Parasassi T, De Stasio G, d'Ubaldo A, Gratton E (1990) Phase fluctuation in phospholipid membranes revealed by laurdan fluorescence. Biophys J 57(6):1179–1186
17. Parasassi T, De Stasio G, Ravagnan G, Rusch RM, Gratton E (1991) Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of laurdan fluorescence. Biophys J 60(1):179–189
18. Parasassi T, Gratton E (1992) Packing of phospholipid vesicles studied by oxygen quenching of laurdan fluorescence. J Fluoresc 2(3):167–174
19. Antollini SS, Barrantes FJ (1998) Disclosure of discrete sites for phospholipid and sterols at the protein-lipid interface in native acetylcholine receptor-rich membrane. Biochemistry 37 (47):16653–16662
20. Jurkiewicz P, Olzynska A, Langner M, Hof M (2006) Headgroup hydration and mobility of DOTAP/DOPC bilayers: a fluorescence solvent relaxation study. Langmuir 22(21):8741–8749
21. Bagatolli LA (2013) LAURDAN fluorescence properties in membranes: a journey from the fluorometer to the microscope. In: Mely Y, Duportail G (eds) Fluorescent methods to study biological membranes, Springer series on fluorescence, vol 13. Springer, Heidelberg

22. Roenneberg T, Dragovic Z, Merrow M (2005) Demasking biological oscillators: properties and principles of entrainment exemplified by the Neurospora circadian clock. Proc Natl Acad Sci U S A 102(21):7742–7747
23. Jaeger J, Goodwin BC (2001) A cellular oscillator model for periodic pattern formation. J Theor Biol 213(2):171–181
24. Kruse K, Julicher F (2005) Oscillations in cell biology. Curr Opin Cell Biol 17(1):20–26
25. Goldbeter A (1996) Biochemical oscillations and cellular rhythms: the molecular basis of periodic and chaotic behaviour. Cambridge University Press, Cambridge
26. Richard P (2003) The rhythm of yeast. FEMS Microbiol Rev 27(4):547–557
27. Richard P, Teusink B, Hemker MB, Van Dam K, Westerhoff HV (1996) Sustained oscillations in free-energy state and hexose phosphates in yeast. Yeast 12(8):731–740
28. Poulsen AK, Lauritsen FR, Olsen LF (2004) Sustained glycolytic oscillations–no need for cyanide. FEMS Microbiol Lett 236(2):261–266
29. Olsen LF, Andersen AZ, Lunding A, Brasen JC, Poulsen AK (2009) Regulation of glycolytic oscillations by mitochondrial and plasma membrane H+-ATPases. Biophys J 96(9):3850–3861
30. Reijenga KA et al (2001) Control of glycolytic dynamics by hexose transport in Saccharomyces cerevisiae. Biophys J 80(2):626–634
31. Novak B, Tyson JJ (2008) Design principles of biochemical oscillators. Nat Rev Mol Cell Biol 9(12):981–991
32. Clegg JS (1984) Properties and metabolism of the aqueous cytoplasm and its boundaries. Am J Physiol 246(2 Pt 2):R133–R151
33. Luby-Phelps K (2000) Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. Int Rev Cytol 192:189–221
34. Fels J, Orlov SN, Grygorczyk R (2009) The hydrogel nature of mammalian cytoplasm contributes to osmosensing and extracellular pH sensing. Biophys J 96(10):4276–4285
35. Spitzer J (2011) From water and ions to crowded biomacromolecules: in vivo structuring of a prokaryotic cell. Microbiol Mol Biol Rev 75(3):491–506, second page of table of contents
36. Hazlewood CF, Chang DC, Nichols BL, Woessner DE (1974) Nuclear magnetic resonance transverse relaxation times of water protons in skeletal muscle. Biophys J 14(8):583–606
37. Yoo H, Paranji R, Pollack GH (2011) Impact of hydrophilic surfaces on interfacial water dynamics probed with NMR spectroscopy. J Phys Chem Lett 2(6):532–536
38. Kiser PF, Wilson G, Needham D (1998) A synthetic mimic of the secretory granule for drug delivery. Nature 394(6692):459–462
39. Fenimore PW, Frauenfelder H, McMahon BH, Young RD (2004) Bulk-solvent and hydration-shell fluctuations, similar to alpha- and beta-fluctuations in glasses, control protein motions and functions. Proc Natl Acad Sci U S A 101(40):14408–14413
40. Slavik J (1982) Anilinonaphthalene sulfonate as a probe of membrane composition and function. Biochim Biophys Acta 694:1–25
41. Zasetsky AY (2011) Dielectric relaxation in liquid water: two fractions or two dynamics? Phys Rev Lett 107(11):117601
42. Thomson JF (1963) Biological effects of deuterium. Pergamon, Oxford
43. Leterrier JF (2001) Water and the cytoskeleton. Cell Mol Biol 47(5):901–923
44. Korn ED, Carlier MF, Pantaloni D (1987) Actin polymerization and ATP hydrolysis. Science 238(4827):638–644
45. Stark BC, Wen KK, Allingham JS, Rubenstein PA, Lord M (2011) Functional adaptation between yeast actin and its cognate myosin motors. J Biol Chem 286(35):30384–30392
46. Frauenfelder H, Fenimore PW, Young RD (2007) Protein dynamics and function: insights from the energy landscape and solvent slaving. IUBMB Life 59(8–9):506–512
47. Frauenfelder H (2008) What determines the speed limit on enzyme catalysis? Nat Chem Biol 4 (1):21–22
48. Frauenfelder H, Fenimore PW, Chen G, McMahon BH (2006) Protein folding is slaved to solvent motions. Proc Natl Acad Sci U S A 103(42):15469–15472

49. Ling GN (1962) A physical theory of the living state: the association-induction hypothesis. Blaisdell, New York
50. Schrödinger E (1944) What is life? The physical aspect of the living cell. Cambridge University Press, Cambridge
51. Young RD, Fenimore PW (2011) Coupling of protein and environment fluctuations. Biochim Biophys Acta 1814(8):916–921
52. Ling G (2007) Nano-protoplasm: the ultimate unit of life. Physiol Chem Phys Med NMR 39 (2):111–234
53. Ling G (1984) In search of the physical basis of life. Plenum, New York
54. Troshin AS (1966) Problems of cell permeability. Pergamon, Oxford
55. Beloussov LV (1989) Dynamical levels in developing systems. In: Goodwin B, Sibatani A, Webster G (eds) Dynamic structures in biology. Edinburgh University Press, Edinburgh