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Invited perspective

The Ubiquity of High-energy Nanosecond Fluorescence in DNA duplexes

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ABSTRACT

During the past few years, several studies reported that a significant part of the intrinsic fluorescence of DNA duplexes decays with surprisingly long lifetimes (1-3 ns) at wavelengths shorter than the $\pi\pi^*$ emission of their monomeric constituents. This **High-Energy Nanosecond Emission** (HENE), hardly discernible in the steady-state fluorescence spectra of most duplexes, was investigated by time-correlated single photon counting. The ubiquity of HENE contrasts with the paradigm that the longest-lived excited states correspond to low-energy excimers/exciplexes. Interestingly, the latter were found to decay faster than the HENE. So far, the excited states responsible for HENE remain elusive. In order to foster future studies for their characterization, this perspective presents a critical summary of the experimental observations and the first theoretical approaches. Moreover, some new directions for further work are outlined. Finally, the blatant need for computations of the fluorescence anisotropy considering the dynamic conformational landscape of duplexes is stressed.

Our understanding of the excited state relaxation in DNA duplexes has made considerable progress since the turn of the century. On the one hand, spectral evolutions induced by UV excitation, could be resolved thanks to femtosecond spectroscopy.¹⁻⁶ On the other, various computational methods brought an increasing insight into the associated processes.⁷⁻¹² Thus, it is widely accepted by now that an important part of the excited state population survives in duplexes much longer (from tens to hundreds of ps) than their monomeric constituents, which decay within a few ps. This behavior has been attributed to the formation of excited charge transfer (CT) states between two stacked nucleobases and associated with excimer/exciplex emission. The latter, reported for duplexes at room temperature already in the 1990s,¹³⁻¹⁴ appears at wavelengths longer compared to monomer $\pi\pi^*$ emission.

In parallel, time-resolved fluorescence experiments performed on longer time-scales revealed unexpected nanosecond components at wavelengths shorter than the monomer fluorescence. Hereafter, we denote them as HENE, for High-Energy Nanosecond Emission, as opposed to LENE, for Low-Energy Nanosecond Emission. A short description of HENE was reported recently in our account on the intrinsic DNA fluorescence. In the meantime, new studies 1, 21-22 rendered the assessment of the excited states responsible for this intriguing emission much more complex than that proposed by the first computational approaches. Therefore, we have decided to resume the current state of knowledge of this poorly understood, yet ubiquitous, fluorescence emission in the present perspective. After presenting an overview of the most striking results, we highlight the importance of HENE relatively to LENE in the steady-state fluorescence spectra of duplexes, discuss potential connections with structural and electronic issues, stress the need of further computational studies and provide experimental tips that are crucial for more refined studies. We hope that this perspective will foster future work, both experimental and theoretical, allowing a more thorough characterization of the excited states responsible for HENE and assess their potential implication in the DNA photochemistry and/or their utilization in optoelectronic nanodevices.

The show case: (dGC)_n•(dGC)_n. The first observation of HENE was reported for polymeric duplexes with alternating GC sequence.¹⁵ Their steady-state fluorescence spectra are largely dominated by a band peaking about 4000 cm⁻¹ higher than that of an equimolar mixture of mononucleotides dGMP and dCMP (Figure 1a) and decaying with a lifetime of 1.3 ns (Figure 1b).

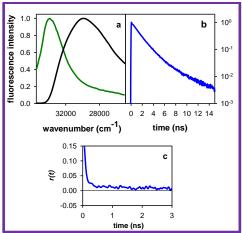


Figure 1. Fluorescence properties determined for $(dGC)_n \circ (dGC)_n$ (green, $n \approx 1000$) in phosphate buffer: (a) steady-state emission spectrum (green) compared with that of an equimolar mixture of dGMP and dCMP (black). ¹⁵ Decay ¹⁵ (b) and anisotropy ¹⁹ (c) recorded at 305 nm.

The influence of a certain number of factors on the $(dGC)_n \bullet (dGC)_n$ fluorescence was inspected and the following conclusions were drawn: (i) A minimum size of about 10 base-pairs is required so that a exponentially decaying HENE band to appear (Figure 2a); for this minimum size, it is more pronounced for hairpins than for duplexes, the base-pairing being less efficient for the latter structures.¹⁸ (ii) The fluorescence quantum yield ϕ of long polymeric duplexes increases upon decreasing the ionic strength,¹⁹ known to augment the persistence length of the duplex,²³ accounting for its stiffness. (iii) It disappears upon B-DNA \rightarrow Z-DNA transition.¹⁹ (iv) The spectral width decreases significantly upon increasing the size of the system;¹⁸ for polymeric duplexes excited at 267 nm it is only 3300 cm⁻¹ (fwhm), versus 7700 cm⁻¹ (fwhm) for an equimolar mixture of dGMP and dCMP (Figure 1a).¹⁵ (v) The fluorescence anisotropy associated with this ns emission, r_{ns} , has a weak positive value (~0.02; Figure 1c).^{15, 18-19} (vi) The fluorescence quantum yield ϕ observed for UVB excitation (7.0×10⁻⁴ at 285 nm) is five times higher than that observed for UVC excitation (1.3×10⁻⁴ at 267 nm).¹⁹

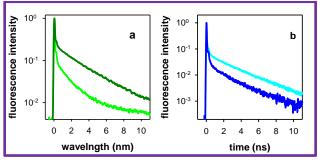


Figure 2. Effect of the duplex size on the normalized fluorescence decays recorded at 305 nm for double-stranded structures in phosphate buffer: (a) $(dGC)_n \bullet (dGC)_n$ hairpins excited at 255 nm (n = 6, light green; n= 10, dark green); ¹⁸ (b) $(dA)_n \bullet (dT)_n$ duplexes excited at 285 nm (n = 20, cyan; n \approx 1000, blue). ²¹

The above spectral properties indicate that HENE stems from collective excited states whose population depends on the initial Franck-Condon states. Because of the static conformational disorder, there is a multitude of excitonic Franck-Condon states, whose energy spans over the absorption spectrum.²⁴ In addition, on the grounds of the measured r_{ns} values (Figure 1c), HENE was assigned to "mixed" excitons resulting from coupling between $\pi\pi^*$ and CT states; the reasoning that lead to this assignment, as well as the associated weak points, will be discussed later.

The hidden HENE. With the exception of $(dGC)_n \bullet (dGC)_n$, HENE is not distinguishable in the steady-state fluorescence spectra of duplexes. It can be assumed that they are masked by the relatively stronger $\pi\pi^*$ emission. To illustrate this, the spectra of four polymeric duplexes are shown in Figure 3 together with those of the corresponding stoichiometric mixture of mononucleotides. Three of the duplexes; $(dAT)_n \bullet (dAT)_n (n \approx 2000)^{20} (dA)_n \bullet (dT)_n^{21} (n \approx 1000)$ and genomic (calf thymus) DNA, have spectra that do not exhibit any particular feature on the blue side of their spectrum, practically overlapping the monomer spectra. Therefore, past fluorescence studies focused solely on the main emission band (around 330 nm and considered to emanate from localized $\pi\pi^*$ states) and the excimer/exciplex emission at longer wavelengths. Surprisingly, more recent time-resolved fluorescence studies have revealed a different picture.

For all the studied duplexes, either with repetitive $^{20-21, 27}$ or disordered 17 base sequence and, most importantly, for genomic DNA, $^{16, 20}$ a ns component was detected on the blue side (305/310 nm) of the spectrum. Its relative contribution to the total number of emitted photons at these wavelengths is far from negligible, varying from $\sim 30\%$ to $\sim 60\%$. This is shown in Figure 3 by vertical bars: the grey ones represent

the total number of photons emitted at the considered wavelength, while the blue ones represent the part of these photons emitted after 1 ns, determined from the TCSPC fluorescence decays as described in reference 6). For comparison, the "ns photons" at 420 nm have also been quantified and are shown as red bars. It can be observed that the "red" photons contribute less to the fluorescence spectra (5 to 8 times) than the "blue" ones. This is particularly striking for $(dAT)_n \bullet (dAT)_n$, whose spectrum exhibits a long wavelength band peaking at 420 nm, assigned to exciplex emission.

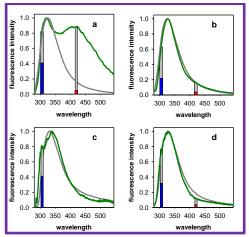


Figure 3. Contribution of HENE (blue) and LENE (red) to the fluorescence spectra of polymeric duplexes (green) in phosphate buffer. Vertical grey bars correspond to the total number of photons emitted at a given wavelength while blue and red bars indicate the part of these photons emitted after 1 ns, as determined from the TCSCP decays. (a) $(dAT)_n \bullet (dAT)_n$; $n \approx 2000$, ²⁰ (b) $(dA)_n \bullet (dT)_n$; $n \approx 1000$, ²¹ (c) $(dG)_n \bullet (dC)_n$; $n \approx 1200^{27}$ and (d) calf thymus DNA. ¹⁶ The spectra of the corresponding stoichiometric mixtures of mononucleotides are shown in grey. Excitation: 267 nm.

The lifetimes, determined from tail-fitting of the 305/310 nm decays on the ns time-scale, fall in the range of 1-3 ns. As for our show-case, $(dGC)_n \bullet (dGC)_n$, the fluorescence anisotropy on the ns time-scale r_{ns} exhibits small positive values (0.01-0.05). Moreover, the number of base-pairs in the examined model duplexes affects the decays, modifying their pattern and/or the relative amplitude of the ns component (Figures 2a and 4). The observed trends are not uniform, but depend on the base sequence. For $(dG)_n \bullet (dC)_n$, which was prepared in small quantities by a specific biochemical technique, 28 only a limited number of experiments could be performed; the size effect was not examined, nor fluorescence anisotropies were determined.

A first theoretical approach. Quantum chemistry calculations, based on the time-dependent density functional theory (TD-DFT) and performed for duplexes with alternating base sequences, $(dGC)_2 \bullet (dGC)_2$ and $(dAT)_2 \bullet (dAT)_2$, brought an important insight in the excited states responsible for the HENE of these systems. They were assigned to $\pi\pi^*$ excitons delocalized over four bases across both strands and characterized by a weak intrastrand charge transfer among a purine and a pyrimidine: $G^{+\delta} \to C^{-\delta}$; $A^{+\delta} \to T^{-\delta}$. Due to their mixed $\pi\pi^*/CT$ character, these states were named HELM, for High-energy Emitting Long-lived Mixed excitons. They were found to originate from specific Franck Condon states already involving several base pairs and presenting a weak mixing between $\pi\pi^*$ and CT states. More specifically, these Franck-Condon states are characterized by coplanar base-pairs for which the twist is the same in both strands. The conservation of these geometrical elements is essential for their evolution toward HELM excitons. In this respect, it is worth-recalling older theoretical studies, carried out in the frame of the

exciton theory taking into account static conformational disorder.^{24, 29} They showed that the Franck-Condon states of $(dGC)_5 \bullet (dGC)_5$ are, on average, more extended than those of $(dAT)_5 \bullet (dAT)_5$ and $(dA)_{10} \bullet (dAT)_{10}$. This finding is related to the existence of three hydrogen bonds per Watson-Crick pair in GC duplexes instead of two in the AT analogs. Thus, the smaller rigidity of the AT duplexes should favor localization of the excitation and subsequent decay via a monomer-like ultrafast channel, explaining why their HENE is bleared by $\pi\pi^*$ emission (Figure 3).

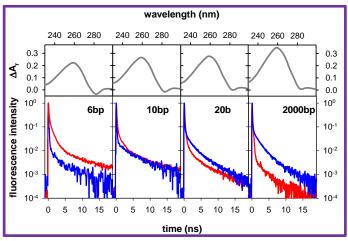


Figure 4. Correlation between the absorption spectra and the long-lived fluorescence of $(dAT)_n \bullet (dAT)_n$ with 6, 10, 20 and 2000 base-pairs in phosphate buffer. Upper panel: thermal differential absorption spectra: $A_r = (A_{96^{\circ}C} - A_{23^{\circ}C})/A_{23^{\circ}C}$. Lower panel: Normalized fluorescence decays recorded at 305 nm (blue) and 420 nm (red) following excitation at 267 nm.

Franck-Condon states and relaxation pathways. A dependence of HENE on the Franck-Condon states, predicted by the above theoretical study, has already been mentioned for $(dG)_n \bullet (dC)_n$, whose ϕ varies with the excitation wavelength.

The experimental study of $(dAT)_n \bullet (dAT)_n$ revealed an additional correlation between the absorption spectra and the long-lived fluorescence. For this base sequence, two hairpins, an oligomeric duplex and a polymeric duplex, composed respectively of 6, 10, 20 and 2000 base-pairs, respectively, were examined.²⁰ A first observation concerns the evolution of the hypochromism exhibited by the absorption spectra. It was quantified by the thermal spectra, obtained by subtracting the absorption spectrum at 23°C ($A_{23^{\circ}C}$) from that at 96°C ($A_{96^{\circ}C}$), after the melting of the duplex, when the electronic interactions among the bases are much reduced. We observe that the hypochromism (upper panel in Figure 4) increases with the number of base-pairs, meaning that the coupling between $\pi\pi^*$ and CT states becomes stronger. In parallel, the patterns of the fluorescence decays recorded at 305 and 420 nm are modified (lower panel in Figure 4). Upon increasing the size of the system, the amplitude of the ns component at the blue side increases, while the decay becomes faster and faster at the red side.

Such a correlation corroborates the assignment of HENE to HELM excitons. As a matter of fact, X-ray diffraction measurements in solution³⁰ as well infrared spectroscopy experiments³¹ showed that a size extension of the duplex reduces the amplitude of conformational motions, rendering them stiffer. Consequently, both the probability for orbital overlap and the conservation of the initial configurations increases, favoring relaxation pathways leading to HENE.

Beyond duplexes. As exposed above, it appears that HENE is ubiquitous in DNA duplexes. The question arises whether a double-stranded structure is a necessary requirement for this type of emission. As a matter of fact, it is has not been observed for polyadenine single strands,³² known to adopt a helical structure, allowing for electronic coupling among bases.³³ This means that a helical geometry alone is not sufficient to give rise to HENE. In contrast, it was detected for a four-stranded structure (G-Quadruplex) formed by association of two identical DNA strands $d(G_4T_4G_4)$ in the presence of K^+ cations.²² For this system, a long-lived fluorescence with a lifetime of 2.1 ns was observed at 315 nm, representing 10.8% of the photons emitted at this wavelength. Nevertheless, no HENE was found in the fluorescence of the other G-Quadruplexes whose decays were recorded at short wavelengths. The latter were formed by either the folding of a single strand $d(G_3AT_2)_3G_3^{34-35}$ or the association of four TG_nT stands (n= 3, 4, 5).³⁶ We will further discuss this aspect later.

From DNA to RNA duplexes. While the fluorescence experiments on duplexes carried out in our group concerned solely DNA systems, a study by Ma and Kwok⁵ focused on the polymeric duplex $(rA)_n \bullet (rU)_n$, which is the RNA analog of $(dA)_n \bullet (dT)_n$ (Figures 2b and 3b). Interestingly, they reported two distinct emission bands decaying on the ns time-scale. The high energy one peaks at 324 nm and is characterized by a lifetime of 1.7 ns. For comparison, that of $(dA)_n \bullet (dT)_n$ occurs at ~305 nm and decays with a lifetime of 2.6 ns.²¹ One striking difference between the HENE of the two systems resides in their anisotropy r_{ns} : that reported for $(rA)_n \bullet (rU)_n$ (~0.15) is much higher than that of $(dA)_n \bullet (dT)_n$ (~0.02). On the basis of these r_{ns} values, the HENE of the RNA duplexes was assigned simply to "excitons", in contrast to that of the DNA duplexes, which had been associated with mixed $CT/\pi\pi^*$ states. However, for both RNA and DNA duplexes, the correlation between the fluorescence anisotropy and the nature of the associated excitons is not straightforward.

The fluorescence anisotropy issue. The assignment of HENE to mixed $CT/\pi\pi^*$ states was established on the grounds of the fluorescence anisotropy r_{ns} , which was found to be in the range 0.02-0.05. To this end, some basic rules determined long ago were taken into account. Hence, it was considered that fluorescence anisotropy r of the $\pi\pi^*$ emission of B-form duplexes is bound to be higher or equal to 0.1.6. The latter value corresponds to transition dipoles randomly distributed within the same plane on which lie the absorption transition dipoles. Such an in-plane depolarization of the fluorescence, detected experimentally, amay result from energy transfer among nucleobases, whose aromatic planes are perpendicular to helix axis. Consequently, r values somewhat lower than 0.1, were attributed to the contribution of an out-of-plane component arising from coupling between $\pi\pi^*$ and CT states. We note that negative r values, corresponding to angles between absorption and emission transition dipole moments higher than the magic angle, were observed for single-, double and four-stranded structures only in the "red" part of the fluorescence spectra on the ps³⁹ and sub-ns^{32, 35, 38} time-scales. Consequently, this emission was assigned to excited states having a strong CT character.

In addition to the CT character of the emitting states, geometrical reasons may also lead to r values lower than 0.1. Conformational disorder associated with parameters such as buckle, tilt, propeller, twist or roll, result in out-of-plane deviations of the bases, and, hence, of the transition dipole moments of their $\pi\pi^*$ transitions. Fluorescence anisotropies associated with pairs of distinct exciton states, built considering only dipolar coupling and taking into account static conformational disorder, were computed.²⁹ Moreover, quantum chemistry calculations, including orbital overlap among bases, reported r values.⁷ But neither of these theoretical studies took into account the statistical nature of the experimental measurements. Such computations encompassing, not only the static structures, but also the dynamics of the conformational motions, from the fs to the ns time-scales, would provide valuable information about the nature of HENE,

and, in a more general way, of the intrinsic DNA fluorescence. Among others, such refined calculations would allow rationalizing the differences in r expected for the same type of excited states in DNA and RNA duplexes, differing in respect to the angle formed between the aromatic planes of the bases and the helix axis.

The mystery of the excited states responsible for HENE. The ensemble of the experimental observations described above point toward collective the emitting states. HELM excitons offer an elegant description of the excited states giving rise to HENE in the case $(dGC)_n \bullet (dGC)_n$ and $(dAT)_n \bullet (dAT)_n$. Still, two points remain to be rationalized through computations: (i) the fluorescence anisotropy issue, developed in the previous paragraph, which will allow checking on solid grounds the extent of the CT character of the emitting states; (ii) an explanation of the longevity of the excited states responsible for HENE. This implies that they are "protected" by an important energy barrier (which needs to be computed) from evolving towards the conical intersections responsible for the ultrafast deactivation of the $\pi\pi^*$ states of nucleic acids. 40 Alternatively, the local rigidity of the duplex could hinder their evolution toward fully developed CT states, both because of conformational constrains and by affecting the dynamics of the water molecules. 41-43 The latter play a key role in the solvation of CT states drawing their energy downwards. To this end, the conformational landscape of the duplex and its hydration shell must be taken into account, which is not an easy task. Such factors have started to be included in the description of excited state relaxation in DNA multimers on the ps scale. 44-45 Hopefully, the continuous progress in computational techniques will allow their extension over longer timescales and larger systems.

In any case, the type of HELM excitons determined by quantum chemistry methods for duplexes with alternating base sequence, involving a weak intrastrand CT interaction between a purine and a pyrimidine, cannot account for the HENE of homopolymeric duplexes. Thespecific structural and electronic features of the corresponding excited states remain to be identified. Experimental studies on other model duplexes, containing, for example, two base-pairs in a repetitive way, such as $(dGA)_n \bullet (dCT)_n$ or $(dGT)_n \bullet (dCA)_n$, in which intrastrand stacking involves different bases, could be informative. And comparison between DNA and RNA duplexes composed of the same base sequence, such as $(dGC)_n \bullet (dGC)_n$ and $(rGC)_n \bullet (rGC)_n$, should allow better assessing the role of geometrical factors vs the electronic ones.

With the existing model of HELM excitons in mind, in which precise structural elements, as well the existence of CT interactions between a purine and a pyrimidine are crucial, we are tempted to make the following remark concerning G-Quadruplexes. The only G-Quadruplex for which HENE was detected so far is a fully symmetric system. Not only it is formed by two identical DNA strands, but also the bases are arranged in a symmetric way. 46-47 First, the guanines adopt an anti-parallel arrangement. Second, we have two identical diagonal thymine loops allowing for orbital overlap between a guanine and a thymine.²² In contrast, no HENE was found for its tetramolecular analog, formed by association of four identical DNA strands d(TG₄T). In the latter structure, the arrangement of the guanines adopt an all-parallel arrangement with a C₄ symmetry axis, ⁴⁸ but the thymines behave as daggling groups at the ends of the strands, without permanent stacking on the guanines, ruling out any symmetric interactions. This observation corroborates the view that an appropriate organization, including two types of bases, is necessary for the appearance of HENE. It could be further tested by varying the types of loops⁴⁹ and/or the peripheral bases located within the loops. Thus, structures with lateral loops⁵⁰ would be interesting, because they preclude CT interactions between guanines of the quadruplex core and the peripheral bases. Alternatively, modification of the type of the peripheral bases [i.e. G-Quadruplexes formed by folding of $(G_3C)_3G_3$ or $(G_3T)_3G_3$ strands] or their number [i.e. $(G_3T)_3G_3$ vs $(G_3T)_3G_3$)] while maintaining the parallel arrangement of guanines in the core,⁵¹ should shade some light the on the factors affecting HENE.

The importance of high energy long-lived excited states. Independently of their exact features, depending on the base sequence geometrical arrangement, the excited states responsible for HENE could play a role in the UV-induced damage of the genetic code and be potentially interesting for optoelectronic devices.

On the biological side, we recall that cyclobutane pyrimidine dimers (CPDs), a major UV-induced DNA lesion,⁵² are formed via $\pi\pi^*$ states;^{12, 53} this reaction was found to occur in thymine single strands within 1 ps.⁵⁴ But the fluorescence of genomic DNA, whose spectrum practically overlaps that of its monomeric constituents, stemming from $\pi\pi^*$ states (Figure 3d), decays on the ns time-scale instead of a few ps for the monomers.¹⁶ This has been interpreted in terms of delayed fluorescence resulting from repopulation of $\pi\pi^*$ states from HELM excitons under the effect of conformational motions, which break their coherence.²⁰ Should this picture be confirmed, the dimerization reaction would take place over much longer times. If in contrast, future studies demonstrate that the excitons responsible for HENE are devoid of CT character, then CPD formation over several decades of time would be straightforward.

On the technological side, the interesting point resides in the high ϕ of HENE. This conclusion is derived from the fact that transient absorption measurements on model duplexes and genomic DNA have not detected any component decaying on the ns time-scale.¹⁻² Considering the sensitivity of such measurements, we judge that the excited states responsible for HENE should represent less than 1% of the excited state population. Yet, the corresponding ϕ , determined by exciting the ensemble of ground state configurations, may be as high as 7.0×10^{-4} , ¹⁹ meaning that their "intrinsic" ϕ of HENE is at least 0.07. The detailed characterization of these excited states should provide a guidance for the design of highly fluorescent DNA/RNA segments and also of G-Quadruplexes, which are more easily programmable than duplexes, for applications in the field of nanotechnology.⁵⁵

Experimental tips for future studies. In order to achieve a better characterization of HENE, not only of those discussed here, but also of other DNA/RNA systems, it is important to consider the following points.

First of all, the purity of the systems is a paramount condition. In the reported studies, DNA oligomers were purified by polyacrylamide gel electrophoresis or reversed phase HPLC and tested by MALDI-TOF; genomic DNA was purified according to the procedure reported in reference 16. Oligomers stemming from various companies were also tested. In addition, the purity of the salts used for the preparation of the aqueous solutions has to be higher than 99.99%. Suspecting that HENE may arise from interaction of some impurity with DNA, different types of salts were systematically experimented and even ultrapure water delivered by two different MILLIPORE apparatus was used. Our conclusion has been that the purer the nucleic acids and the ingredients of the solution, the more blue-shifted the florescence spectrum is. In other terms, emission from impurities tend to appear as LENE and may have been erroneously attributed to excimer/exciplex fluorescence in the past.⁵⁶⁻⁵⁷ The same remark is valid for fluorescence stemming from DNA damaged during the experiments by the laser pulses. Examples are shown in the SI of references 15 and 16. In order to avoid this caveat, it is crucial to reduce the excitation intensity (typically, excitation intensities of ~3 KWcm⁻² were used), continuously stir the solution, and renew it as often as possible (preferably working with a continuous flow of fresh solution). A compulsory control is to record/analyze successive signals and, only if they do not change during the measurement, merge them to increase the signal-to-noise ratio.

A second key point is the sensitivity of the fluorescence detection. In order to get a better grip on the long-lived blue-shifted emission in DNA/RNA systems, new measurements with better spectral resolution

and higher sensitivity in this spectral region (<305 nm) are required. In parallel, it is mandatory to also perform systematic studies while varying the excitation wavelength.

Extension to other spectroscopic techniques. In addition to improved fluorescence experiments, other time-resolved techniques could shed light on these elusive "nanosecond" excited states. As mentioned previously, the existing transient absorption studies on DNA duplexes have not revealed any significant ns signals. However, it is worth noticing that, with only one exception, these studies probed the induced transient absorption or the ground state bleaching at single wavelengths. It is possible that by extending the probed spectral domain, in particular towards the near-infrared, where the fingerprint of these high-energy states may show up, as well as the excitation wavelength, could prove helpful. Also by extending the transient absorption measurements to several ns valuable new information could be obtained. This could be achieved by using asynchronous optical sampling methods, 58-59 and a right-angle pump-probe configuration, which allow probing longer detection path-lengths with a diminished time-resolution. Finally, one could further speculate that new techniques, such as 2D electronic spectroscopy and attosecond XUV spectroscopy, already applied on DNA components 60-62 could open up completely new directions, keeping in mind the recommendations about the sample handling and the excitation procedure outlined above.

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