In the letter “Base-Stacking Heterogeneity in RNA Resolved by Fluorescence-Detected Circular Dichroism Spectroscopy” Widom & Hoeher describes the RNA structure-related fluorescence properties of common nucleobase analog 2-aminopurine (2PA). The Authors use fluorescence detected circular dichroism to resolve the fluorescence properties of 2PA in stacked and unstacked positions in RNA sequence oligonucleotide.

The manuscript is well written and reads good but since the Authors use the FDCD which is not a first choice technique to study RNA/DNA heterogeneity I’m strongly missing the TCSPC data. The Author states at the end of the manuscript that lifetimes are planned to be measured, but I think it is essential to incorporate at least some initial decays of the studied system in this Letter. Referring to lifetimes in the literature is not enough in this case.

The results section starts with the sentence:

Page: 5, line 22: “(...)2-AP dinucleotide exhibited 9.7-fold fluorescence quenching relative to 2-AP riboside (Figs. 1B and S1), indicating either that it occupies a conformation in which 2-AP has a 9.7-fold lower quantum yield, or that it exists in equilibrium between conformations with quantum yields that are higher and lower than that value”.

If it “exists in equilibrium between conformations with quantum yields that are higher and lower than that value” (and most probably it does, looking at the presented FDCD results) there should be an explicit confirmation in lifetime components distribution.

Especially that Authors often refer to von Hippel et. al work from 2004 which revealed only 2 components in lifetime decay of 2-AP in DNA. It indicates that the system is relatively easy to interpret. I think measuring the TCSPC in the Authors’ system will improve the manuscript, allow to anchor the results in the field, and make a smooth introduction of the FDCD technique for structural analysis of nucleic acids.

In general, I think FDCD seems to be a valuable addition to the toolkit for studies on nucleobases analogs photophysics and it is worth publishing in JPC Lett.
Minor suggestions to consider for the Authors:

- Authors can consider moving the theoretical part about the FDCD into SI to make the presentation of the results more consolidated.

- Page 8, line 17 “(...) the presence of multiple structures that interconvert on a timescale slower than the fluorescence lifetime.” If the time scale is slower maybe microsecond time-resolved spectroscopy could be useful? See the example:

Koch, Marius, Roxana Nicolaescu, and Prashant V. Kamat. "Photodegradation of polythiophene-based polymers: excited state properties and radical intermediates." The Journal of Physical Chemistry C 113.27 (2009): 11507-11513.

Reviewer: 2

Comments to the Author

The authors use a combination of fluorescence detected and standard circular dichroism spectroscopy to investigate stacking configurations in RNA labeled with the modified base 2-aminopurine (2-AP). They find that the fluorescence is only from the unstacked configurations, suggesting that use of this reporter as a fluorescence probe may be limited to a subpopulation of the heterogeneous RNA configurations. While these findings may guide future use of this reporter, experiments in larger RNA structures along with further quantification and explanation would clarify the usefulness of these properties.

Specific comments:

1) To make these results more useful to the community, the authors should measure how the stacked and unstacked configurations behave within larger scale (5-10 bases) and hybridized structures.

2) As noted by the authors, examination of base stacking is often performed with emission lifetime measurements. If possible, further characterizing the samples with time or frequency resolved fluorescence lifetime measurements would make the quantification here more useful to the community.

3) While the authors interpret their results within a two-state model of either stacked or unstacked bases, they should include discussion of “intermediate” partial stacked structures that could confuse this picture results.

4) The authors should address the physical rationale for the stacked and un-stacked configurations from an energetics perspective. For example, are the recovered population ratios on par with what is expected given the relative energies of each stable structure. Furthermore, is rapid switching between these states expected to occur at room or physiological temperatures?

5) While the inclusion of ethanol does promote unstacking of bases, this also leads to changes in the viscosity and local dielectric constant. The authors should quantify the extent to which these effects may impact their solvent dependent results.

Author’s Response to Peer Review Comments:
We thank the reviewers for their helpful comments and are happy to see that they found the research reported in our manuscript to be of high quality and broad interest. Below, we outline the changes we have made in this revision. Changes are highlighted in the copy of the manuscript provided under “Supporting Information for Review Only”.

**Major changes**

As requested by both reviewers, we have performed time-correlated single-photon counting experiments on the samples discussed in the manuscript. As the reviewers anticipated, this allowed us to make useful connections to the literature and added nuance to the sorting of structures into “unstacked” and “stacked” ensembles. The TCSPC results are discussed throughout the text and reported in a new Table 1, Figure S2 and Table S1.

**Responses to specific reviewer comments**

**Reviewer 1:**
- Authors can consider moving the theoretical part about the FDCD into SI to make the presentation of the results more consolidated.

  In response to this suggestion, we elected to shorten the theory section considerably, removing the material that does not directly pertain to the data analysis presented in the manuscript. We believe that the results section flows more naturally now, as the reviewer suggested that it would. We left the presentation of the 2-state model in its entirety because, unlike the general theory section, it is not simply a summary of previously published work.

- Page 8, line 17 “(...) the presence of multiple structures that interconvert on a timescale slower than the fluorescence lifetime.” If the time scale is slower maybe microsecond time-resolved spectroscopy could be useful? See the example:

  Koch, Marius, Roxana Nicolaescu, and Prashant V. Kamat. "Photodegradation of polythiophene-based polymers: excited state properties and radical intermediates." The Journal of Physical Chemistry C 113.27 (2009): 11507-11513.

  The methods used in the cited paper are potentially promising for the study of base stacking. A probe with a longer excited state lifetime (potentially one that can access a triplet state like those discussed in the cited paper) would need to be used in order to obtain transient absorption signals as such long delays. In our manuscript, we cite two papers that used different microsecond-resolved techniques (single-molecule FRET and temperature-jump infrared spectroscopy) to study base stacking kinetics.
Reviewer 2:

1) To make these results more useful to the community, the authors should measure how the stacked and unstacked configurations behave within larger scale (5-10 bases) and hybridized structures.

   While the potential of applying FDCD to larger systems and more complex structures is very exciting, we believe that such measurements are beyond the scope of this manuscript. Significant publications have focused entirely on small model systems like the ones we studied, such as:

   Somsen, van Hoek and Amerongen, Fluorescence Quenching of 2-Aminopurine in Dinucleotides. *Chem. Phys. Lett.* 2005, 402, 61–65.

   Jean and Hall, Stacking–Unstacking Dynamics of Oligodeoxynucleotide Trimers. *Biochemistry* 2004, 43, 10277–10284.

   These publications were later cited by numerous others that investigated more complex systems such as telomeric DNA (Gray et al., *Biochemistry*, 2010, 49, 179-194) and DNA-protein interactions (Greiner et al., *Biochemistry*, 2015, 54, 6012-6020 and many more).

   Taking into consideration the precedent exemplified by works like the ones cited above, the addition of TCSPC measurements in this revision, and the concise format of *J. Phys. Chem. Lett.*, we believe that the manuscript is of appropriate scope in its revised form.

2) As noted by the authors, examination of base stacking is often performed with emission lifetime measurements. If possible, further characterizing the samples with time or frequency resolved fluorescence lifetime measurements would make the quantification here more useful to the community.

   See the “Major changes” section above.

3) While the authors interpret their results within a two-state model of either stacked or unstacked bases, they should include discussion of “intermediate” partial stacked structures that could confuse this picture results.

   We now discuss this factor during the initial introduction of the 2-state model and in the discussion section.

   Introduction of 2-state model:

   “This step sorts the continuum of structures potentially adopted by an oligonucleotide into an ensemble of unstacked conformations with zero CD signal at the wavelengths being considered, and an ensemble of stacked conformations that contribute the entire CD signal.”

   Discussion:

   “The 2-state model presented here sorts intermediate structures into stacked or unstacked ensembles based on whether they contribute to the CD spectrum. In the dinucleotides studied
here, the “zero CD” ensemble includes both unquenched and partially quenched subpopulations identified through TCSPC. In contrast, in C(2-AP)C, the prevalence of the least quenched subpopulation alone is comparable to (in buffer) or larger than (in 30% EtOH) the entire “zero CD” population (Tables 1 and 2).

4) The authors should address the physical rationale for the stacked and un-stacked configurations from an energetics perspective. For example, are the recovered population ratios on par with what is expected given the relative energies of each stable structure. Furthermore, is rapid switching between these states expected to occur at room or physiological temperatures?

Stacking free energies have previously been determined for RNA dinucleotides, and we now address their implications in the discussion section:

“Free energies determined through NMR (44,45) and temperature-dependent UV spectroscopy (46) show that stacking is slightly disfavorable in rArC and rCrA, while molecular dynamics simulations predict it to be slightly favorable. (47) Assuming that 2-AP exhibits similar stacking thermodynamics to A, this is consistent with the assertion above that the ensemble of unstacked conformations encompasses more structures and is more populated than the minor unquenched conformation observed in lifetime measurements. For C(2-AP)C, the free energy change resulting from a transition from the stacked to the unstacked structure depicted in Fig. 4A can be very roughly estimated by adding together the enthalpies of unstacking of rArC and rCrA, and averaging their entropies of unstacking and multiplying by 1.5 (because complete trinucleotide unstacking liberates three bases rather than two). Using values from ref. (46), \( \Delta G = \Delta H - T \Delta S \) yields a rough estimate of \( \Delta G_{\text{unstack}} = +2.1 \text{ kcal/mol at } 298 \text{ K} \), corresponding to an unstacked population of 3%. This suggests that our model’s estimate of 6% is a reasonable value based on stacking energetics.”

Rapid switching is expected to occur at room and physiological temperatures. The original submission cited a previous publication that demonstrated sub-millisecond stacking and unstacking dynamics at room temperature. We have added a second citation when kinetics are mentioned in the introduction, and we revisit the subject of kinetics in the discussion.

Introduction:

“Bases fluctuate between stacked and unstacked conformations on a microsecond timescale (9,10), and these transient structures may provide targets that are kinetically trapped by ligand or protein binding (11–13).”

Discussion:

“Three exponential decay components were observed in 2-AP-labeled DNA trinucleotides dXd(2-AP)dX, but the slowest was only ~3.5 ns at 20 °C, significantly shorter than the lifetime of free 2-AP. It was concluded that 2-AP persists in a fully unstacked conformation for less than 10 ns, with fluctuations back to stacked conformations "gating" decay to the ground state. (42) Slower stacking dynamics on the timescale of microseconds have been observed through single-molecule FRET (9) and temperature-jump infrared spectroscopy. (10) Gating appears to be less significant in C(2-AP)C, which exhibits a slowest decay
component of 7.1 ns in aqueous buffer. The lifetime of this component decreases to 6.4 ns in 30% ethanol despite an increase in viscosity that would be expected to slow the stacking/unstacking kinetics, suggesting that gating is not responsible for the reduction in lifetime.”

5) While the inclusion of ethanol does promote unstacking of bases, this also leads to changes in the viscosity and local dielectric constant. The authors should quantify the extent to which these effects may impact their solvent dependent results.

In response to this comment, we have added the following text when the experiments in ethanol are first introduced: "Addition of ethanol at this concentration increases the viscosity of pure H2O from 0.89 \times 10^3 \text{ Pa s} to approximately 2 \times 10^3, (38) and decreases its dielectric constant from 78.41 to approximately 66. (39) It has previously been shown that a decrease in dielectric constant of this magnitude impacts the morphology of double-stranded DNA condensates (40) and promotes folding of certain RNA species, (41) potentially by enhancing counterion condensation around the phosphate backbone. This is not expected to be a dominant factor for our short single-stranded oligonucleotides, which have only 1-2 phosphate groups per molecule.”

We revisit the topic of viscosity in the discussion, when the “gating” observed in previous time-resolved studies of DNA trinucleotides is discussed: “Gating appears to be less significant in C(2-AP)C, which exhibits a slowest decay component of 7.1 ns in aqueous buffer. The lifetime of this component decreases to 6.4 ns in 30% ethanol despite an increase in viscosity that would be expected to slow the stacking/unstacking kinetics, suggesting that gating is not responsible for the reduction in lifetime.”

We thank the reviewers for their helpful comments, which have helped us to improve the manuscript considerably in this revision.

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