## **COMMENTS**

## Comment on "Limits on Fluorescence Detected Circular Dichroism of Single Helicene Molecules"

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In his paper, "Limits on Fluorescence Detected Circular Dichroism...", Ohen makes some rather sweeping and dismissive claims regarding chiroptical spectroscopy of single molecules in general, and our previously published work in particular. As the name of one of us (M.D.B.) appears in the acknowledgments without advanced knowledge or consent, we feel an obligation to comment directly. It is true that Prof. Cohen contacted M.D.B. prior to submission of the manuscript, to which a detailed response was sent shortly after. We were certainly grateful for the opportunity to view the manuscript prior to publication; however, most of our comments and suggestions for revision were largely ignored. Thus, the fact that we were acknowledged in the paper is troubling in that it implies that we (collectively) agree with the main conclusions of this paper.

The central issue is distortion of the circular polarized excitation field at the sample plane induced by dichroic filters commonly used in fluorescence microscopy of single molecules. It is well-known that ellipticities in excitation polarization can generate linear dichroism in fluorescence that could appear as large circular dissymmetries in fluorescence excitation. As Cohen demonstrates, interrogation of the polarization state of a circular polarized laser can be significantly distorted ( $\approx 50-60\%$  induced ellipticity) upon reflection from a dichroic filter. We have long understood this effect, and any implication or direct claim that we were ignorant or naïve with respect to such distortions (and the inherent artifactual results that could be generated in an experiment) is certainly not true. In both our published papers<sup>2,3</sup> (refs 1 and 18 in the Cohen paper), the polarization state was well characterized, and control runs were made with dye-doped polymer nanospheres as well as for achiral linear dipole molecules to quantitatively assess any artifactual response. This has already been well documented.

In our typical mode of polarization tuning, we looked at the retroreflection of a circularly polarized input beam into the microscope and assessed the degree of ellipticity via the extinction of the retroreflected beam (which changes polarization sense on reflection). As Prof. Cohen called that into question in the current paper, we interrogated directly the fidelity of the circular polarization state of the laser after reflection from the dichroic mirror (Omega Optical XF2027-

485DRLP), and at the sample plane with both the dichroic mirror and objective in place. We first prepared a circular (right or left) polarized excitation beam by placing a linear (vertical) polarizer followed by an achromatic quarter waveplate oriented at ±45°; the purity of the input circular polarization was determined to be 98.8% by passing the light through another quarter waveplate and looking at the intensity ratio in H/V basis of the retroreflected beam. We then looked at the fidelity of the circular polarization as the beam was reflected off the dichroic mirror alone, which gave a circular polarization purity of 98.7%. Finally, we measured the polarization of the beam, as it was reflected from the dichroic mirror and through the objective, to be 98.7%. This number was established by two detection methods; first, by converting the circular polarization back to linear polarization and looking at the intensity ration of H and V components using a second quarter waveplate and analysis of H and V intensities using a rotating linear polarizer, and second, by resolving the H and V components using a Wollaston prism. The results were the same to within experimental error. Thus the induced distortion in the circular polarization of our excitation source through the microscope (all optics included) is of order 0.1%—not 40%, as suggested in the Cohen paper. Thus, our experiments are based on a circular polarization that is not dissimilar from Cohen's using his defined compensation approach generating CPL at the sample.

The second issue regards the breadth and shape of the single-molecule dissymmetry parameter distributions. In Figure 3B, the results shown by Cohen for CPL generated at the sample span a ( $\approx 3\sigma$ ) range of  $\pm 0.5$ . In our published results for triaryl amine helicene excited at 457 nm, approximately 90% of the probability density is contained in the range of g-values of  $\approx \pm 0.7$ , which is not grossly dissimilar from Cohen's result obtained from CPL at the sample. Cohen points to shot-noise, blinking, and photobleaching as the main contributors to the width of his observed distributions, which again is at odds with our own observations. It is (obviously) true that fluorescence intermittency and photobleaching limit information content in any single-molecule measurement. As we pointed out in our original paper, we looked closely at control distributions of both multichromophoric polymer nanosphere samples, as well as linear (achiral) dipole systems to construct a kind of "instrument response function" for our experiment. Both those control distributions were symmetric about g = 0 and had similar widths (fwhm  $\approx 0.2$ ). In our results, we sampled molecules with long enough photochemical lifetime to observe (with several exposures for a particular excitation polarization) intensity variations for at least 1.5 R/L excitation polarization cycles. As we pointed out in our private communication with Prof. Cohen (but not mentioned in his paper), we believe that the principal difference between these two sets of data lie in different subpopulations of the molecules sampled. We (necessarily) selected molecules with high photostability so that we could have long enough exposure times (A) to integrate out any short-time intensity fluctuations (blinking, etc.) and (B) to rotate through multiple right-left cp excitation cycles so that we get information on what (if any) fluctuations in the dissymmetric response from a single molecule.

In our view, this paper certainly has merit both in the defined polarization compensation method that he uses and in its cautionary tone. However, to dismiss explicitly our published work as linear dichroism artifacts is not justified.

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## References and Notes

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