

Excitation Spectra And Stokes Shift Measurements Of Single Organic Dyes At Room Temperature

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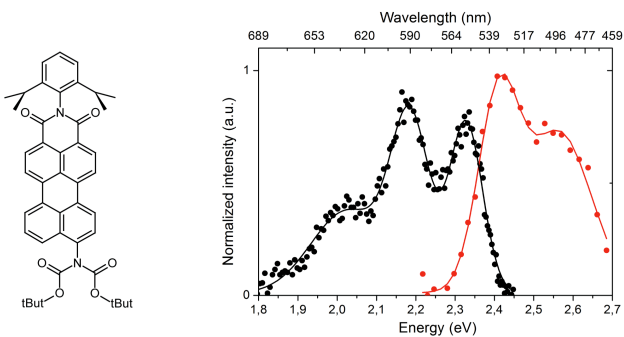
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ABSTRACT: We report measurements of excitation and emission spectra of single, polymer-embedded, perylene dye molecules at room temperature. From these measurements we can derive the Stokes shift for each single molecule. We determined the distribution of excitation and emission peak energies and thus the distribution of single molecule Stokes shifts. Single molecule Stokes shifts have not been recorded to date, and the Stokes shift has often been assumed to be constant in single molecule studies. Our data show that the observed spectral heterogeneity in single molecule emission originates not only from synchronous energetic shifts of the excitation and the emission spectra, but also from variations in the Stokes shift, speaking against the assumption of constant Stokes shift.

TOC GRAPHICS



KEYWORDS. Single Molecule, Spectroscopy, Photophysics, Fluorescence, Excitation, Spectral heterogeneity, Perylene

Single molecule fluorescence spectroscopy has become a widely used technique to yield unprecedented insights into molecular properties and interactions in a wide range of problems.¹⁻⁹ Single molecule spectroscopy has also given unique insights into the photophysical properties of the emitters themselves,¹⁰⁻¹⁵ by avoiding ensemble averaging effects. Most of the photophysical parameters that can be probed at the ensemble level have become accessible at the single molecule level, including the evolution of intensity over time,¹⁶ the lifetime,¹⁷ the polarization,¹⁸⁻¹⁹ and the emission spectra²⁰ of single emitters. Recently Orrit and coworkers demonstrated in pioneering work the detection of a single molecule based on photothermal contrast due to the molecule's absorption.²¹

To record an absorption spectrum, the fraction of the incident light that gets absorbed by the sample needs to be determined per excitation wavelength. This is an exceedingly challenging exercise since the absorption of a single molecule is very low compared to the incident light. However, we recently developed a method to record excitation spectra from a single emitter,²² which gives access to the absorption band maximum, since absorption and excitation spectra generally coincide. In this report we demonstrate for the first time to the best of our knowledge the recording of excitation and emission spectra of the same single organic fluorophore at room temperature. Using the excitation and emission spectra of the single molecules, we are able to measure the Stokes shift of single molecules.

The Stokes shift is the difference in energy between the band maxima of the absorption and the lower-energy (red-shifted) emission, and is a key parameter of fluorescence.²³ Practically, the Stokes shift is crucial to any use of fluorescence-based methods, since it allows the easy separation of emitted fluorescence from excitation light by using appropriate optical filters. The loss in energy between excitation and emission is caused by non-radiative dissipation processes

that are characteristic for the fluorophore. The Stokes shift is also affected by excited state processes. For example, if the dipole moments of the excited state and ground state differ, the Stokes shift will increase with solvent polarity (Mataga-Lippert plots),²³ due to solvent relaxation processes after excitation. On the ensemble level, the Stokes shift has proven to be a sensitive parameter reporting on the polarity, mobility and flexibility of the nanoenvironment of the fluorophores.²⁴

The Stokes shift also plays an important role in the phenomena of spectral diffusion and spectral heterogeneity observed in room temperature single molecule spectroscopy. In essence, the spectral position of the emission of single fluorophores that interact with their environment varies from emitter to emitter and fluctuates over time.^{10, 20, 25-33} From ensemble studies it is well known that the spectral properties of a fluorophore, that is the absorbance as well as the emission, strongly depends on external factors like solvent polarity, viscosity, the presence of certain ions, etc.³⁴ The observed single molecule spectral heterogeneity has been attributed to heterogeneity in the conformation of macromolecules, and to variations in the guest-host coupling between the fluorophore and its nanoenvironment that determine the exact photophysical parameters of the fluorophore. However, it remains an open question whether the observed variations in single molecule spectral emission positions arise from environment-induced synchronous shifts of the absorbance and emission spectra, or from differences in the Stokes shift between emitters, or from combinations of both. In the absence of detailed insights, a number of studies have used the “constant Stokes shift assumption”,^{25-26, 35} that is, that the Stokes shift is not affected by the differences in the nanoenvironment.

For our study we chose the perylene derivative N,N-di-(tert-butoxycarbonyl)-9-amino-N-(2,6-diisopropylphenyl)perylene-3,4-dicarboximide embedded in a polystyrene matrix. The emission

spectra of polymer-embedded single perylene molecules have been found to show molecular heterogeneity and spectral diffusion.²⁶⁻²⁸ Perylenes are known to be exceptionally photostable, allowing us to acquire both an excitation and an emission spectrum from the same molecule to determine the Stokes shift. The intrinsically high photostability of perylenes also minimizes possible biasing of data due to selection and analysis of a subpopulation of only the most photostable fluorophores. The ensemble spectra acquired in a toluene solution show distinct peaks (see Figure 1) that are characteristic for perylenes. The Stokes shift, by definition the energy difference between the excitation peak with lowest energy and the emission peak with the highest energy, is 96meV for the ensemble solution.

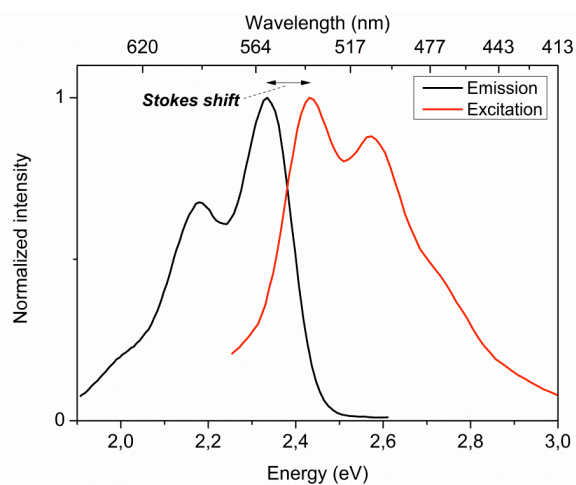


Figure 1. Ensemble excitation and emission spectra of perylene dissolved in toluene. The excitation and emission spectra show characteristic peaks. The Stokes shift is by definition the energy difference between the excitation peak with the lowest energy and the emission peak with the highest energy. The Stokes shift of the studied perylene derivative in toluene is determined to be 96meV.

For the single molecule studies we embedded perylene at a low concentration suitable for single molecule detection in a thin film of polystyrene (for details on sample preparation and instrumentation see ²² and Supporting Information). For the single molecule excitation spectra we swept the excitation wavelength from 2.2eV to 2.75eV (461nm to 561nm) and recorded the emission intensity per excitation wavelength in a detection band around 582 nm. As excitation source we used a supercontinuum white light source in combination with an acousto-optic tunable filter to select the desired excitation. For single molecule emission spectra we used excitation at 510 nm and detection via a prism based spectrometer and an EMCCD camera.

In this study, we successfully recorded 100 excitation and 80 emission spectra from single perylene molecules. We discarded spectra that were not complete. Due to blinking and bleaching of emitters, not all excitation and emission spectra were fully acquired. In particular, the excitation spectra are vulnerable to blinking, because these spectra are essentially time-series of the emission intensity measurements at different excitation wavelengths. Furthermore, we discarded datasets showing the signature of a spectral jump, which are unusual broadening of the emission spectra and sudden jumps in the recorded excitation spectra. Although spectral jumps rarely occur,²⁷ these jumps might bias our data.

Representative examples of single molecule excitation and emission spectra are shown in Figure 2. For all recorded single emitter excitation and emission spectra we observe the characteristic shape expected from the ensemble spectra, but show molecule-to-molecule variations in spectral positions and shapes. The variations in the spectral shape reflect molecule-to-molecule differences in the Franck-Condon factors for excitation transitions, representing the excitation analogue to the previously observed spectral variations in the emission spectra.²⁷ The variations in the excitation spectral position relate to differences in the energy gap between

ground state and excited state. Note that alterations of the Stokes shift, related to relaxation and reorientation of the nanoenvironment, after the excitation event, do not contribute to the distribution of excitation spectral positions.

For the emission spectra (Figure 2b), we observe heterogeneity in spectral positions as well as in the shape of the spectra in agreement with previous reports.^{26-28,36} Analogous to the differences in the excitation spectral shapes, the differences in spectral shape of the emission spectra reflect differences in the Franck-Condon factors for the emission transitions. The emission spectral position however depends on the energy gap between ground state and excited state upon excitation, as well as on the Stokes shift due to the radiationless dissipation and loss of energy due to possible reorganization of the fluorophores nanoenvironment when in the excited state.

To quantify the spectral variations, we constructed histograms of the energies of the lowest energy excitation peaks of all 100 successfully recorded excitation spectra (Figure 2c), and of the highest energy emission peak energies of all 80 successfully recorded emission spectra (Figure 2d). The mean peak energies of the excitation and emission distributions are 2.38eV and 2.25eV, respectively. These energies are slightly lower than the spectral peak energies of the ensemble spectra, which are at 2.43eV and 2.34eV. We do not observe equivalent changes in the spectra from bulk samples of high concentrations of perylene embedded in a thick film of polystyrene (see Supporting Information). We speculate that the differences in Stokes shift we observe between solution and single molecule experiments arise from the embedding of the molecules in a thin, spin-coated film of polystyrene. As expected, both peak energy distributions appear normally distributed, which was confirmed using statistical methods (see Supporting Information).

The widths of the distributions can give initial insights into whether the Stokes shift between the molecules is constant or varies from molecule to molecule. One would expect the widths of the energy distributions of the excitation maximum and the emission maximum to be identical in the case that the Stokes shift is constant for all molecules. However, if the Stokes shift is not constant for different molecules, one would expect additional broadening of the emission distribution. Variable Stokes shifts, e.g. due to additional relaxation processes characteristic for the individual molecule that change the energy of the excited or ground state while the molecule is in the excited state, will add to the distribution sampled in excitation, such that the emission distribution will be wider than the excitation distribution.

We find the characteristic signature of molecule-to-molecule variations of the Stokes shift in our data. The width (2σ) of the excitation distribution is 63 meV and hence significantly smaller than the width of the emission distribution, which is 95 meV. The distribution widths of the excitation and emission peak energy show the exact energy gaps between the ground and excited state for the excitation and emission process. The larger distribution width of the emission peak energy histogram compared to the distribution width for the excitation spectra, however, shows that there is an additional contribution from processes that are related to the excited state that contribute to the spectral variations of the emission spectra. This difference in the distribution widths strongly indicates that the Stokes shift of the single emitters shows variations from molecule to molecule.

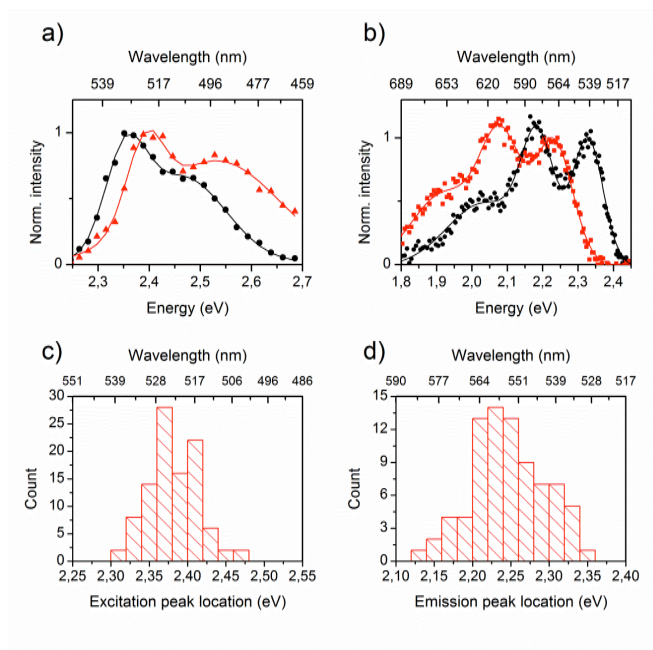


Figure 2. Examples of recorded excitation (a) and emission spectra (b) from different emitters. Solid lines are a guide to the eye, based on double and triple Gaussian fits to the data. For both the excitation and the emission spectra we observe molecule to molecule differences in the shape and the spectral position of the recorded spectra. The peak position histograms for the excitation (c) and emission (d) show the distribution of the peak positions.

Using our data we can also directly study correlations of excitation and emission spectral positions and determine the Stokes shift for single emitters. For 59 molecules we obtained complete excitation and emission spectral datasets of the same molecule, from which the Stokes shift of the single molecule could be determined. Typical spectral datasets are shown in Figure 3a.

The spectral heterogeneity is clearly visible in the excitation and emission spectra as the peak energies vary from emitter to emitter. Interestingly, the excitation and corresponding emission spectra in Figure 3a (top three panels) appear to be shifting synchronously in the same direction

of energy, while the Stokes shift does not show noticeable differences for these different fluorophores. However, for some molecules we find stronger deviations in the Stokes shift (see lowest panel). To examine the correlation between excitation and emission spectral shifts we plot the excitation peak energy versus the emission peak energy for all successfully recorded full spectral datasets (Figure 3b). We observe a clear trend that higher excitation peak energies are commensurate with higher emission peak energies. The data points globally follow a line with a slope of 1 (dashed line as guide to the eye in Figure 3b) in the scatterplot. Were the data points to strictly follow a line with slope 1, this would be evidence of a constant Stokes shift. However, the data points form a cloud rather than a line, hinting at a contribution from an additional factor, which is likely to be related to the distribution of the Stokes shift.

We then determined the Stokes shift for each of the single molecules from the excitation and emission spectral peak energies, with an estimated uncertainty of 10meV, and constructed a Stokes shift histogram (Figure 3c). We find the Stokes shift to be distributed around 128 ± 39 meV (sd). This is in reasonable agreement with the Stokes shift that is found for the ensemble spectra (96meV). The Stokes shift histogram clearly shows the distribution of the Stokes shift, reflecting differences in the polarity and flexibility of the individual nanoenvironment that contributes to the Stokes shift. It is thus the combination of both variations in the energy gap between the ground state and the excited state and variations in the Stokes shift that result in spectral variations observed in the emission spectra.

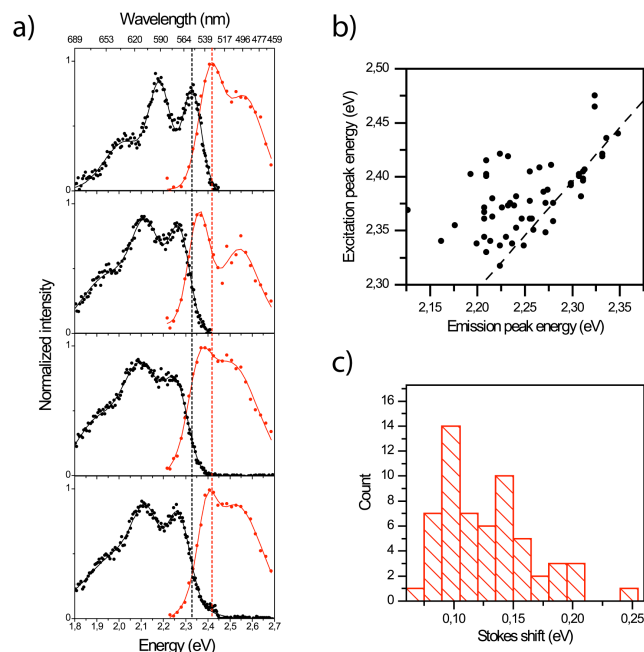


Figure 3. (a) Spectral datasets of four different single emitters. The intensities are normalized by maximum intensity and filtered using a Gaussian filter. The emission and excitation spectra are fitted with triple and double Gaussian functions, respectively, as guides to the eye (solid lines). The dashed lines indicate the emission (black) and excitation (red) peak energies of the upper spectral dataset, providing a guide to the eye to show the spectral shifts. (b) Excitation peak energy versus emission peak energy. The dashed line has a slope of 1 and is a guide to the eye indicating a synchronous shift between excitation and emission peak energies. (c) Stokes shift distribution.

For the samples studied here, the Stokes shift does not follow a normal distribution as we would have expected. We perform a S-W normality test on the Stokes shift distribution, which gives a p-value of 0.002, showing that the Stokes shift distribution is significantly different from a normal distribution. Cluster analysis (see Supporting Information), suggests three different groups. The peaks in the Stokes shift histogram might represent different, preferred, dissipation

pathways that can be formed by the perylene embedded in polystyrene. We do not find a clear correlation between excitation maximum and Stokes shift (see Supporting Information) that would be indicative of differences in the local polarity causing the differences in Stokes shift. We hence speculate that differences in the local flexibility give rise to the observed differences in the Stokes shift.

In conclusion, we have determined single emitter excitation spectra and Stokes shifts at room temperature for the first time to the best of our knowledge. Our data gives new insights into the phenomenon of spectral heterogeneity of single molecule fluorescence emission spectra. We find a distribution of spectral positions of the excitation spectra, providing evidence of molecule-to-molecule differences in the energy gap between ground and excited states. We further find a distribution of the Stokes shifts at the single molecule level, related to differences in the excited state relaxation before emission, which we attribute to local variations in the polarity and flexibility of the emitter's nanoenvironment. The differences in the Stokes shift are further supported by our observation that the excitation peak energies show a smaller distribution than the emission peak energies. Clearly, spectral variations in the emission spectra and the often observed spectral diffusion originate on the one hand from differences in the energy gap between the ground state and the excited state, and on the other hand from differences in the Stokes shift. We anticipate that our results hold for all matrix-embedded single molecules, including fluorescent proteins for which the protein scaffold forms the embedding matrix. Finally, we believe that detailed characterization of single molecule Stokes shifts may be a promising tool to study local variations in a matrix, for example, in polymers.

ASSOCIATED CONTENT

Supporting Information. Experimental details and statistical data analyses. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Excitation Spectra And Stokes Shift Measurements Of Single Organic Dyes At Room-Temperature

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Supporting Information

Experimental details

Sample preparation

To prepare our samples we diluted the perylene derivative N,N-di-(tert-butoxycarbonyl)-9-amino-N-(2,6-diisopropylphenyl)perylene-3,4-dicarboximide in a solution of 0.8 g polystyrene (PS) in 100 ml of toluene to achieve relevant single emitter concentrations (~1nM). This dilution is spin-coated onto a clean coverslip for 10 seconds at 6000rpm to immobilize the emitters in a PS polymer matrix. The coverslip was cleaned beforehand with an ozone cleaner (*UV/Ozone ProCleaner Plus, Bioforce, San Diego, CA*) for at least one hour.

Experimental procedure

We raster scan the sample with a custom-built single molecule fluorescence setup as explained in detail in reference ¹ to localize the single isolated emitters. All experiments are performed at room temperature. We use an excitation wavelength of 510nm and an intensity of approximately 1kW/cm² at the diffraction limited focus spot, and integrate 10ms per pixel to collect emission from the single perylene dyes across a 10x10μm² area. The perylene dyes typically show a stable emission pattern with a sparse amount of blinking (not shown). After locating the single emitters, we select the ones that are fully spatially isolated and record both the excitation and emission spectra at the same location. To do so, we use two filter sets that can be interchanged in-between the spectral recordings. To measure excitation spectra we use an SP01-561RU filter in combination with LP02-561RU and FF01-582/15 filters (all filters from Semrock Inc., NY, USA) and excite the emitters using a wavelength scanning range from 461nm to 561nm (2.2eV to 2.75eV). The excitation spectra are recorded as a series of total emission intensity measurements with 200ms integration time each, for each excitation wavelength. The excitation wavelengths are tuned using an acousto-optical tunable filter (AOTF, Crystal Technologies, West Chester PA, USA) to select any desirable wavelength in the visible spectral band from a supercontinuum laser source (SC400-PP, Fianium Inc, UK). To measure

emission spectra we use an FF02-447/60 filter in combination with a BLP01-488R long-pass filter (Semrock Inc., NY, USA and excite the emitters at a wavelength of 470nm, and record the emission from ~500nm and longer wavelengths (<2.5eV), using a custom-built prism spectrograph that is equipped with an Andor Newton 971-BV camera (Andor Technology Ltd., UK). The filter sets ensure that excitation and emission light are fully isolated from each other and do not hinder the detection of the emission light coming from the single emitters.

After recording the spectra, we correct the emission spectra for background (recorded at areas between the single emitters). The excitation spectra are also corrected for background (recorded at areas between the single emitters). In addition we apply a photon flux density correction,

$$\Phi_d = \frac{P}{E_{ph} \cdot A} \sim \frac{P(\lambda)}{\lambda}$$

where Φ_d is the photon flux density, P the power at wavelength λ , E_{ph} the energy of a photon and A the area of the diffraction-limited focus spot. We did not correct for wavelength dependent differences in the sensitivity of the setup, which explain some of the differences in the spectral shape of the bulk emission spectra and the single molecule spectra.

Statistical analysis

Normality tests

Both excitation and emission peak energy distributions appear normally distributed, which we would expect, since random fluctuations of molecular properties cause spectral variations that are typically characterized by a normal distribution of the observed quantity.² As an additional test, we apply a Shapiro-Wilk (S-W) normality test to both distributions. The S-W test specifically suitable for small sample sizes ($n \leq 50$), but can also range up to $n = 2000$.³ Generally, a p-value<0.05 for the tested distribution is considered as a proof that the distribution is significantly different from a normal distribution while a p-value of 1 indicates a perfect match with a normal distribution.⁴ For the distribution of excitation peak energies we find a p-value of 0.35 and for the distribution of the emission peak energies a p-value of 0.63, meaning that the S-W test does not consider both distributions to significantly differ from a normal distribution.

The Stokes shift histogram does not appear to be normally distributed. Therefore, we perform a S-W normality test on the Stokes shift distribution, which gives a p-value of 0.002, showing that the Stokes shift distribution is significantly different from a normal distribution.

Statistical analysis of the Stokes shifts

Interestingly, if we analyze the Stokes shifts using a k-means cluster analysis,⁵ a good agreement is found with the Stokes shift histogram when having three statistically distinguishable groups. The mean Stokes shifts of those three groups are 96 ± 12 meV, 142 ± 12 meV and 198 ± 20 meV (standard deviation), which matches the peak locations energies and peak widths at figure 3c. In addition, we select the data points in figure 3b that correspond to these groups and determine the linear correlation coefficient (Pearson's) of the data points within each group. We observe a strongly increased correlation between the excitation and emission peak locations energies for the grouped datapoints ($r_1=0.95 \pm 0.02$, $N_1=29$; $r_2=0.93 \pm 0.05$, $N_2=21$; $r_3=0.85 \pm 0.10$, $N_3=9$), compared to that of all datapoints taken together ($r_{all}=0.59 \pm 0.08$, $N_{all}=59$), even though the statistical sample decreases. The correlation coefficient errors (bootstrapped⁶, see figure S1) of the first two groups also become smaller after subdivision,

supporting the increased correlation of the selected datapoints for the two most prominent peak in the Stokes shift histogram.

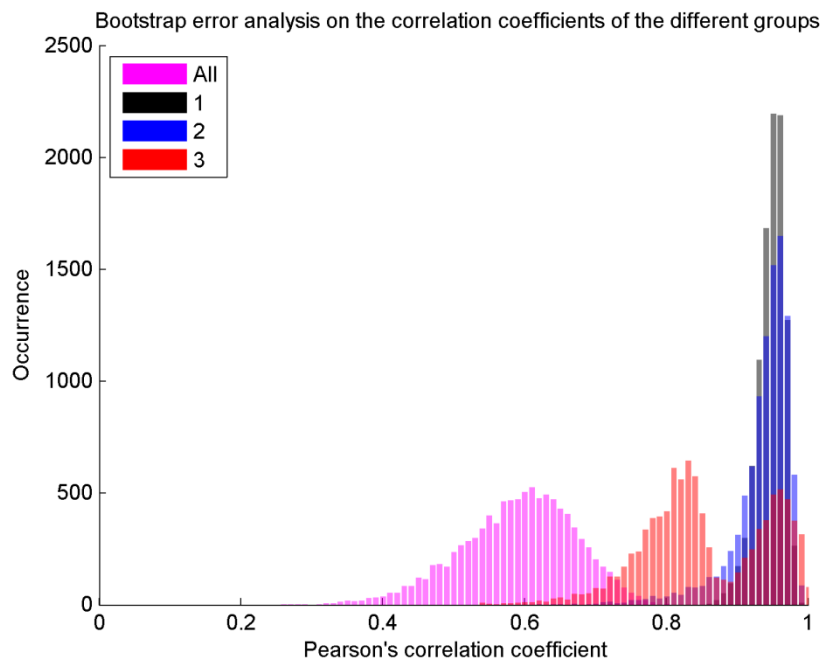


Figure S1 Bootstrap error analysis on the correlation coefficients of the different groups. After grouping the datapoints according to the selection found by the k-means cluster analysis, the correlation coefficient strongly increases and the error becomes smaller, even though the statistical sample decreases.

Additional bulk spectra

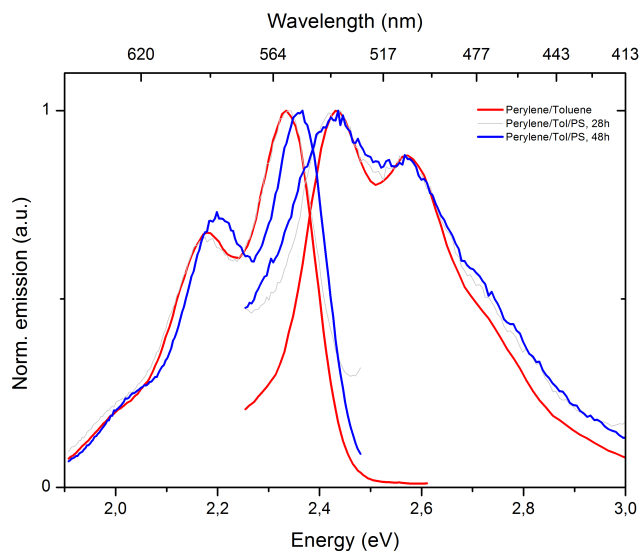


Figure S3 Additional bulk spectra of high concentration perylene in a thick layer of polystyrene upon drying of the layer.

Stokes shift as a function of the excitation maximum

For positive solvatochromism one would expect that the red shift of the excitation maximum is accompanied by an increase of the Stokes shift. We therefore plotted the Stokes shift as a function of the excitation maximum (see below). Interestingly we do not find the clear correlation between excitation maximum and Stokes shift that would be indicative of differences in the local polarity causing the differences in Stokes shift. It is hence likely that differences in the local flexibility give rise to the observed differences in the Stokes shift. A nanoenvironment that does not allow for reorientation to accommodate the changes of the dipole moment in the excited state will give rise to a smaller Stokes shift than a nanoenvironment that allows reorientation.

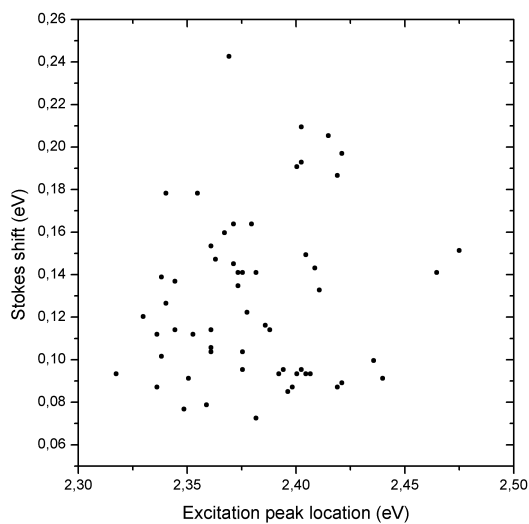


Figure S3 Scatterplots where Stokes shift is plotted versus excitation peak energies. There is no clear correlation between Stokes shift and excitation peak energy.

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