Quantifying Resonant Raman Cross Sections with SERS

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We propose a method based on surface-enhanced Raman scattering (SERS) to estimate the resonance Raman cross sections of dyes. The latter are notoriously difficult (or impossible) to obtain by normal (spontaneous) constant wave Raman spectroscopy when the fluorescence quantum yield of the molecules is good and the overwhelming effect of fluorescence masks the Raman spectrum. We propose here to use the fluorescence quenching occurring in SERS conditions to overcome simply this problem. The principles of the method are described and its limitations discussed in detail. The method is demonstrated by estimating the resonance Raman differential cross sections for Rhodamine 6G for seven different excitation wavelengths across the visible range.

I. Introduction

The determination of Raman cross sections[1,2] is a textbook-like example of molecular spectroscopy.[3,4] However, differential resonant Raman cross sections ($d\sigma/d\Omega$) in molecules are, in general, very difficult (and sometimes impossible) to obtain with normal (spontaneous) Raman spectroscopy. Despite their great importance in techniques such as surface-enhanced Raman scattering (SERS),[2] there is only a scant amount of experimental information on them.[5] The predominant reason for this is the simultaneous generation of fluorescence under resonance conditions in constant-wave (CW) spectroscopy. For fluorescent dyes (which constitute a very important group of probes for SERS in general,[2,6] and for single-molecule SERS in particular[7–10]) the bare fluorescence cross section of the molecules ($d\sigma_{fluo}/d\Omega \sim 10^{-16}$cm^2/sr) typically outstrips the corresponding Raman ones in resonance ($d\sigma_{Raman}/d\Omega \sim 10^{-24}$cm^2/sr) by figures in the range of $8-10$ orders of magnitude. This is of course the primary reason why the field of single-molecule fluorescence[11,12] was developed a lot earlier than its counterpart in Raman spectroscopy. Despite the fact that these cross sections refer to spectrally integrated quantities (i.e., integrated over all emission wavelengths) and that Raman signals are typically much "sharper" than fluorescence emission by a factor of $\sim 10^2-10^3$, this is still not enough to compensate for the remaining $7-8$ orders of magnitude in efficiency between the two: Raman signals are basically "swamped" in the fluorescence background of the spectrum. There are exceptions to the rule though in CW spectroscopy. The most common of which comes from dyes with a poor quantum yield (Q) for fluorescence emission.[2,12] One example of the latter is the molecule crystal violet (CV),[5] with a very low fluorescent quantum yield $Q \sim 5 \times 10^{-5}$ due to internal nonradiative relaxation mechanisms[12,13] (the twisting of the arms of the molecule) in the excited state. Hence, in cases like CV it is indeed possible to measure the CW differential Raman cross section under resonance conditions, by comparing the signal of a known concentration of CV molecules in solution to a reference standard[3,5] under identical experimental conditions. These cases are, nevertheless, more the exception than the rule

for many important dyes. Paraphrasing a recent review article by Anne Myers-Kelley:[14] "Resonance Raman scattering largely places the experimentalist at nature's mercy. If the Raman spectrum is weak or extremely complicated, or sits on top of a strong fluorescence background, there is not much that can be done about it".

A notable exception to the deadlock imposed by the presence of underlying fluorescence has arisen in the past few years in the form of broadband femtosecond stimulated Raman spectroscopy (FSRS); pursued mainly by Mathies and co-workers.[15–19] FSRS is insensitive to background spontaneous fluorescence,[18] and its theory has also been well developed.[17] For many years, a historical predecessor of FSRS to unravel the Raman spectrum under resonance conditions has been the technique of Kerr-gated time-resolved resonance Raman spectroscopy.[20] The early time-resolved methods[21] were mainly based on the idea of measuring the Raman spectrum before the fluorescence arrives; that is, in the window of a few nanoseconds (typically) defined by the lifetime of the excited state.[2,12] However, quantifying the differential cross section is still one step above the mere "extraction" of the Raman spectrum from the fluorescence background, for it implies a normalization with respect to a known standard that can be difficult to achieve in the time-resolved versions of the technique. The best evidence of how difficult it is to obtain reliable values of resonant Raman differential cross sections is perhaps the fact that the first published values in the literature for Rhodamine 6G (arguably one of the best known and most widely used dyes in laser spectroscopy) were published for the first time in 2008 by Mathies and co-workers for a single probe excitation line at 532 nm (doubled Nd:YAG laser), using FSRS.[15] The progress made in FSRS in the past few years is truly impressive, but it still remains a technique that is practiced by a handful of groups around the world. To the best of our knowledge, there are a priori no obvious alternatives in plain CW Raman spectroscopy for the task.

On the theoretical front, quantitative predictions of resonant Raman cross sections are also challenging since they require detailed modeling of excited state dynamics. It is only recently that reports of theoretical predictions have appeared. An example particularly relevant to this study is ref 22, where time-dependent

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density functional theory is used to predict resonant Raman cross sections of Rhodamine 6G. A value of $\approx 5 \times 10^{-26}$ cm^2/sr is obtained for the 1511 cm^{-1} mode. Arguably, such theoretical predictions remain subject to potentially large errors.

In this paper, we propose a new method to quantify experimentally resonant Raman differential cross sections by using the SERS effect and CW Raman spectroscopy. Like any method, it has intrinsic limitations, but in those cases where it can be applied it provides an easy alternative to time-resolved spectroscopy. It can produce experimental estimates for several excitation lines, and it can be used directly with CW-Raman systems that are most commonly available in many laboratories around the world (compared to the availability of time-resolved systems). In the following, we explain the basics of the method and discuss its potential limitations. We then apply it specifically to obtain experimental estimations of dσ/dΩ for RH6G for seven different excitation lines across the visible range.

II. Principles of the Method

A. Basic Concept. One important aspect of surface-enhanced spectroscopy is the interplay between radiative and nonradiative components of the enhancement factor, and how they affect (differently) Raman and fluorescence processes.[2,13,23–25] We review here briefly the basic concepts (with the aid of Figure 1) that we need for the present problem and refer the interested reader to the specialized literature for the details.[2,13,23–25] In a nutshell, Raman profits from radiative enhancements in both the incoming (laser) and Stokes-scattered photon, while fluorescence only profits from an absorption enhancement in the first step of the process. The emission in fluorescence is further limited by the competition between radiative and nonradiative decay from the excited state, the rate of which are modified by different mechanisms in close vicinity to a metal surface. As a result, for molecules adsorbed directly on the metal, the Raman signal is typically enhanced by several orders of magnitude more than the fluorescence signal. This phenomenology is often simplified by simply stating that "fluorescence is quenched while Raman is enhanced" as the molecule approaches the metal surface; this is illustrated schematically in Figure 1a–c and treated in considerable more detail in the specialized literature.[2,13,23–26] In reality, fluorescence may still be enhanced when a molecule is directly adsorbed on the metal,[2,26] but the fluorescence to Raman ratio is indeed strongly quenched.

Using this well-known phenomenology, we can qualitatively envisage the following method to measure an unknown resonance Raman cross-section: we first take a reference molecule of which we know the bare Raman differential cross sections for some modes, and place that molecule in a specific place on a metal surface where it is subject to a Raman enhancement (i.e., we can measure its SERS spectrum). We then take a resonant molecule that we do not know the Raman differential cross sections of (because it was hidden by fluorescence) and place it in the exact same position as the previous one. Thanks to fluorescence quenching, we can again measure its SERS spectrum under identical experimental conditions as the reference. If both molecules are not intrinsically modified by the presence of the metal and both are subject to the same SERS enhancement, simple ratios of peak intensities yield the differential Raman cross sections of the second molecule.

B. Potential Limitations. There are several problems with this simple-minded approach, but all of them admit some sort of a solution or a partial solution. We list a few of the most obvious here:

(1) The method implies that there is no substantial modification of the molecule as a result of its interaction with the surface,
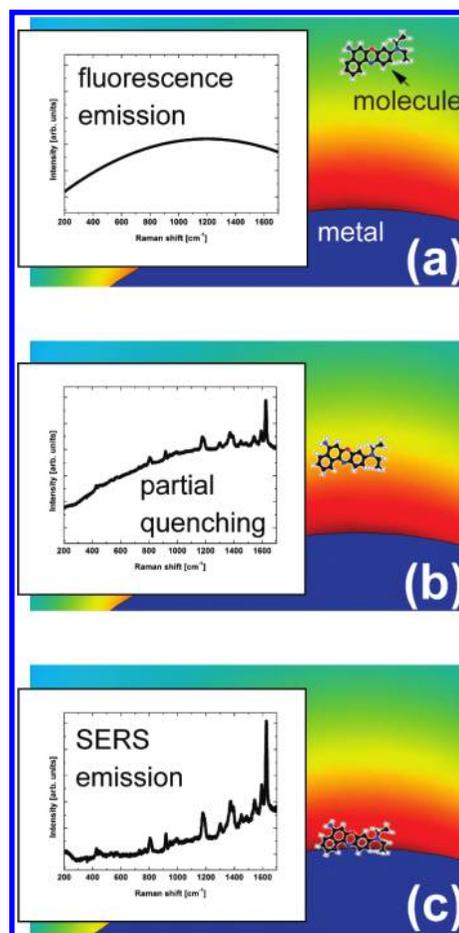
Figure 1. (a) The emission far away from a metallic surface is dominated by fluorescence when the quantum yield of the dye is good. As the dye approaches the surface in (b), both Raman and fluorescence are enhanced; but while Raman always profits from radiative enhancement, the fluorescence emission is a compromise between what is gained in enhanced absorption and what is lost is nonradiative emission to the metal.[2] This is the well-known "quenching of fluorescence" effect seen in SERS. When the molecule is in direct contact with the metal, as in (c), the fluorescence is mostly quenched (with respect to SERS) by nonradiative processes. If we were able to compare two molecules (one with a known and another with an unknown dσ/dΩ) on the same spot on the surface, we would have a (comparative) measure of Raman differential cross sections without interference from fluorescence. The conditions under which this is possible are explained in the text.

that is, that there is no metal-molecule complex formed in the process. This aspect is closely related to the possibility of an additional "chemical enhancement" in SERS.[27] If there is a metal–molecule complex present, then the differential cross section we measure is not that of the bare molecule but rather that of the molecule in the presence of the metal (which can have its resonance condition changed in the process). Although this is a potential limitation, there is some reassurance that such a situation can in principle be detected by making measurements on several different dyes, over a large wavelength range, and ideally on two types of SERS substrate (i.e., silver and gold).

(2) The simple-minded approach as stated above implies that we are able to position the molecule and the reference molecule in exactly the same place. This is obviously unachievable, and it is exacerbated by the well-known drastic inhomogeneities of the enhancement factor in typical SERS substrates.[28] Instead we can rely not on single-molecule signals but on average properties of the enhancement factor for a given substrate.[5] For as long as the total concentrations of dyes are small enough to avoid mutual interference on the surface coverage, and large

Quantifying Resonant Raman Cross Sections with SERS

J. Phys. Chem. A, Vol. 114, No. 17, 2010 **5517**

enough to ensure reproducibility of signals over the chosen integration time, we can take average values as representative. The underlying assumption here is that the average SERS EF (or analytical SERS EF[2,5]) is similar for both dyes. We need, for example, to ensure that both dyes have the same adsorption properties on the metal surface (for example by choosing both dyes with a strong affinity to the metal surface).

(3) The idea also assumes that there will be no additional experimental limitation to obtain the SERS signals of both dyes. However, the unequal photobleaching rates for both dyes at a given excitation wavelength can make a difference in the comparison of relative intensities.[29,30] Photobleaching problems can be avoided by working with the lowest possible power densities and their presence can be checked experimentally by performing measurements at two or more different incident laser powers.

(4) In theory there is no limitation on how different the two differential Raman cross sections to be compared can be. In practice, however, it is best to be able to to see both signals within the same dynamic range of the detector without having to change the experimental conditions (increasing power, integration time, etc.,). This implies that we should try to use dyes with similar cross sections. If we are trying to measure a resonant dye, we then need to find another resonant dye with known cross section. Accordingly this second reference molecule cannot be anything but a resonant dye with a poor quantum yield. This enhances the importance for this application of resonant dyes with low (or negligible) quantum yields. Some standard dyes (such as CV) fall into this category, but they can also be engineered if necessary by the addition of quenching moieties to a given chromophore. The choice of the "reference molecule" is an important part of the problem here, and it needs to be resolved on a case-by-case basis depending on the compatibility with the other molecule and the spectral range where we want the comparison to be made.

(5) SERS substrates tend to have problems with reproducibility. A rule of thumb is always that the smaller the enhancement, the better the reproducibility of the signal.[31] Substrates that sustain high enhancements ($\sim10^9$) capable to see single molecules are typically the least reproducible. Part of this issue can be solved by working with low magnification objectives and long integration times to increase sampling range in space and time, respectively (this has the additional benefit of reducing photobleaching issues). But to completely avoid this problem, it is even better to have both dyes simultaneously in the sample. This is similar in some ways to the bianalyte SERS technique,[32] which was developed for single molecule detection. Here, the method is used at much higher analyte concentrations to compare average signals over the same enhancement factor distribution.

(6) Different surface selection rules[33,34] for different molecules could pose a problem, because the electromagnetic SERS EF may vary from mode to mode and from molecule to molecule by a factor related to the orientation of the molecule on the surface. Except for extreme cases (where a peak disappears, for example) this should not contribute much except by a relatively small factor.[34] The cross sections determined with this method are therefore subject to uncertainties in the surface-selection rules factor,[2] which unless independently measured can be assumed to be of the order of a factor of ~2.[34]

Accordingly, the method proposed here is nothing but a bianalyte SERS measurement, where the aim is not to use a contrast signal to detect single molecule cases but rather make a comparison of average signals over the same enhancement factor distribution. This is done for two different resonant molecules: one that we know the Raman differential cross sections of, and one that we do not. Note that we use SERS substrates here to profit not so much from its enhancing properties of the Raman signals (SERS) but mainly for its quenching properties of the fluorescence.

The main underlying assumption is that both molecules experience the same analytical SERS EF.[5] This implies in particular that they have the same adsorption efficiency during sample preparation, that they do not exhibit extreme surface selection rules effects, and no chemical modification or chemical enhancement. The approximate validity of these assumptions (say within a factor $\approx2-3$) is, we believe, sufficiently common (see ref 5 for examples) but must nevertheless be assessed on a case-by-case basis. In particular, measurements on different types of SERS substrates or with different preparation procedures, with different pairs of analytes, and at different excitation wavelengths should all contribute to confirm, or infer, the validity of these assumptions. In fact, as we shall see, there may in some cases be wavelengths for which the Raman cross sections of the unknown dye can also be measured via standard methods (for example when it is in preresonance conditions). This then provides a further independent check of the validity of the proposed method.

With some limitations (summarized in the list above) a simple estimate of resonant Raman cross sections is feasible without resorting to time-resolved spectroscopy. The limitations are real, but there is no technique that is completely free of limitations in that sense. FSRS, for example, also faces limitations in terms of the photostability of the molecules under intense pulsed excitation and the actual Raman gain that can be obtained by stimulation. There will be examples where a quantitative determination of resonant differential cross sections with FSRS will not be possible. With these provisos in mind, when the technique proposed here is applicable, it does provide a very simple estimation of resonant differential Raman cross sections.

III. Experimental Section

A. Experimental Details. As an example of its applicability, we now provide estimations of resonant Raman differential cross sections for Rhodamine 6G (RH6G) at seven different excitation wavelengths in the visible (458, 488, 514, 532, 568, 633, and 647 nm) by a direct comparison with CV.

Raman spectra were taken with a triple-subtractive Jobin-Yvon Raman microscope equipped with a N_2-cooled CCD detector. Different excitations lines in the blue−red region (458, 488, 514, 532, 568, 633, and 647 nm) were obtained from either a HeNe or tunable Ar^+-Kr^+ laser. The triple-subtractive spectrometer is ideal for resonance studies, for it does not rely on the use of notch filters. To eliminate errors stemming from the anisotropic detection efficiency of our spectrometer we measured in a parallel/parallel scattering configuration for all laser lines. We also measured the response of the system in this configuration and took it into account in the cross-section analysis (see Section S.I. of the Supporting Information).

We first measured the bare differential Raman cross sections of CV, summarized in Table 1, using standard methods (see Section S.I. of the Supporting Information for details). Then, we measured ratios of SERS signals of these two dyes in four (very) different types of SERS substrates: Ag colloids in solution, silver films, Au films, and Klarite substrates. In principle, the nature of the SERS substrate should not influence the results for as long as: (i) the substrate does not interfere with the dyes, and (ii) there is enough signal to observe both

TABLE 1: Differential Raman Cross Sections σ (Units $\times 10^{-26}$ cm²/sr) for CV and RH6G[a]

λ_{ex} [nm]	458	488	514	532	568	633	647
σ_{CV}	4.6	9.7	14.4	28.5	67.5	5.1	1.7
σ_{RH6G}	8.9			230[15]		0.22	0.095
$\sigma_{RH6G, SERS}$	5.2	25.6	69.6	149	118	0.64	0.12

[a] The values $\sigma_{RH6G, SERS}$ were obtained by multiplying $\sigma_{CV}(\lambda_{ex})$ with the ratio factor $R(\lambda_{ex})$ obtained from the bi-analyte SERS spectra as explained in Section S.III. of the Supporting Information.

dyes (the reference and the probe). It is a good practice, however, to compare the ratios of Raman signals under SERS conditions in several different substrates. This is done to ensure that there was nothing extremely peculiar about one particular choice of substrate and that similar results can be obtained irrespective of the exact nature of it. If this is the case, we can have more confidence that what we are measuring is an intrinsic property of the probe, and not a property of the probe in combination with the substrate. If the substrate is completely innocuous to the ratios of cross sections between an unknown probe and a reference, then it should not even matter whether we use silver or gold (the two most widely used SERS substrates) as enhancing/quenching materials.

The first series of samples are prepared in solution with Ag Lee and Meisel colloids[35] at 15 mM KCl.[36] Samples were prepared to a total dye concentration of 10 nM (CV+RH6G) by successive dilutions from reference samples at 100 μM. To avoid dilution errors in this process, we obtained the final concentration in three steps. Initially we go from 100 μM to 1 μM, where an absorbance measurement is carried out to ensure proper dilution. At this point the dyes are mixed in a 1:1 ratio and further diluted by a factor of 50 in the colloid, which is then aggregated with KCl. A low magnification ($\times 20$ immersion objective index-matched to water) is used to improve averaging over the sample and avoid photobleaching as much as possible. The spectra are acquired over total integration times of at least 100 s, and the analysis is performed on the average spectra (examples of measurements are provided in the Supporting Information).

In addition to colloid measurements, we check the relative ratios of SERS signals for the dyes deposited at ~100nM concentration on flat silver and gold films and on commercial Klarite substrates (made out of gold). Silver (30 nm) and gold (200 nm) films were deposited on glass slides using a conventional evaporator equipped with a calibrated thickness monitor. Klarite substrates were used as received without further preparation. The dyes (and dye mixtures) were drop casted on the films and/or Klarite substrate and dried under a mild heat. Raman spectra were taken in this case with a $\times 10$ objective (to improve spatial averaging and avoid photobleaching as much as possible) in air, by again using the T64000 Jobin-Yvon triple spectrometer and integration times of ~20−60 s. We made averages over at least 20 different points on the sample to ensure the signal was representative of the average over the substrate. The data on gold substrates tends to be weaker than that on silver films, and it is likely that part of the enhancement seen on silver and gold films comes from surface roughness. For gold substrates we could only obtain spectra at three of the six laser wavelengths we used for silver; this is because the signal is too weak and cannot be quantified in the other cases.

B. Experimental Results. For the analysis, we concentrate only on two modes: 1510 cm⁻¹ of RH6G and 1620 cm⁻¹ of CV simply because they do not overlap too much with other modes and can be easily measured within the same spectral
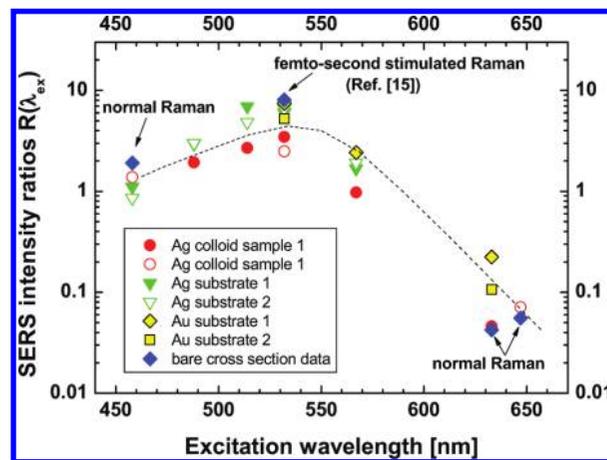

Figure 2. Ratios $R(\lambda_{ex})$ of SERS intensities of the RH6G and CV peak (1510 and 1650 cm⁻¹, respectively) as measured in the bianalyte experiment on different substrates (for details see S.III). The blue, plain diamonds correspond to ratios of bare cross sections $\sigma_{RH6G}/\sigma_{CV}$ (see Table 1): The values at 458, 633, and 647 nm are obtained from our CW Raman spectroscopy measurements in the region outside the main resonance (where Raman signals can be seen above fluorescence). The value at 532 nm is obtained from the FSRS measurement (ref 15) and our own determination of the resonant cross section of CV at that wavelength. The dashed line is only a guide to the eye. From these ratios, $R(\lambda_{ex})$, and from the differential cross section of CV $d\sigma_{CV}/d\Omega$ (inferred by comparing its bare signal to a 2B2MP reference sample, see Section S.I. of Supporting Information), the differential cross sections of RH6G $d\sigma_{RH6G, SERS}/d\Omega$ in Table 1 can be determined.

window of the CCD. The procedure to accurately extract the relative intensities (i.e., the ratio of differential Raman cross-section) of the 1510 cm⁻¹ for RH6G and 1620 cm⁻¹ of CV from an average SERS spectrum is described in Section S.III. of the Supporting Information. In short, we first measure SERS spectra from each of the two dyes and use these as references for a linear least-squares fit of the bianalyte spectrum. This latter step provides a reliable decomposition of the mixture into its binary components and is not affected by asymmetric peaks shapes or peak overlaps (whereas a fit to individual peaks would be). The fit to individual Raman peaks (1510 cm⁻¹ of RH6G and 1620 cm⁻¹ of CV) is only carried out on the reference single-dye spectrum where overlaps with peaks of the other dye are avoided; see Section S.III. for more detail.

Figure 2 summarizes all the SERS intensity ratios of these two peaks for different substrates. This is shown together with ratios of bare cross section measurements done with normal (CW) Raman spectroscopy in regions outside the main absorption of RH6G (where we can see the Raman signal above the fluorescence background). One additional value is inferred from the bare cross section of RH6G measured by FSRS in ref 15 at $\lambda_{ex} = 532$ nm and our own determination of the resonant cross section of CV at that wavelength. To a very good approximation, a consistent picture is obtained for the ratios of cross sections across different substrates and different excitations. From these ratios and the bare $d\sigma/d\Omega$ values of CV (measured directly against a standard as described in Section S.I.), we can obtain estimates for the differential cross sections of the 1510 cm⁻¹ mode of RH6G at different excitation wavelengths. The obtained differential cross sections $d\sigma/d\Omega$ for RH6G and CV are summarized simultaneously in Table 1 and in Figure 3.

IV. Discussion and Conclusion

With several provisos in mind (that need to be evaluated on a case-by-case basis) we have shown here that it is indeed
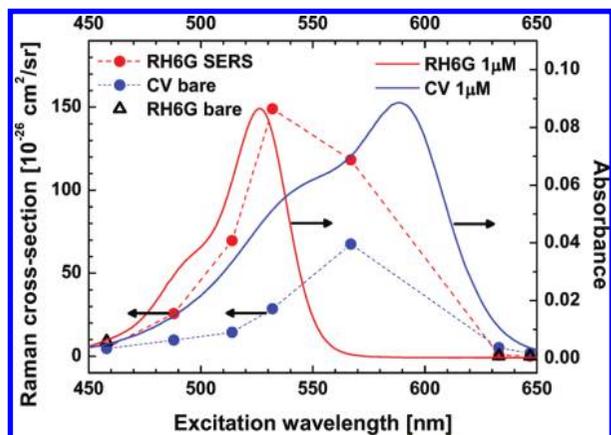
Figure 3. Differential Raman cross sections $d\sigma/d\Omega$ in units of $\times 10^{-26}$ cm^2/sr for the 1620 cm^{-1} mode of CV and the 1510 cm^{-1} mode of RH6G at seven different excitation wavelengths (458, 488, 514, 532, 568, 633, and 647 nm). The data is presented together with the absorption spectra of both dyes. RH6G displays a much stronger preresonance enhancement of the Raman cross section compared to CV which seems to follow the absorption spectrum more closely.

possible to obtain estimates of resonant differential Raman cross sections of dyes with SERS. Using the "quenching" properties of metals for the fluorescence emission of resonantly excited dyes, we can obtain estimates by comparison with a standard affected by the same enhancement distribution. We provide in Figure 3 and Table 1 what we believe is the most complete set of values of resonant Raman differential cross sections for RH6G at seven different excitation wavelengths. Our values at 532 nm compare well with that obtained by the more sophisticated (and naturally more complicated) technique of femtosecond stimulated Raman sprectoscopy.[15] By the same token, we provide the wavelength-dependent resonant differential cross sections of CV in Table 1 and Figure 3 (and the corresponding depolarization ratios in Table S.I.), which were not reported before in the literature, to the best of our knowledge.

Note that RH6G seems to show a much stronger preresonance enhancement of the Raman cross section compared to CV, in which the Raman resonant profile copies rather faithfully the dispersion of the absorption. In addition, the fact that the ratios of cross sections for the bare molecules at 647, 633, and 458 nm (blue data points in Figure 2) show some reasonable agreement with what happens for the same ratio on different substrates (within the limited natural experimental accuracy of these studies) goes some way toward addressing one of the issues raised earlier, that is, the fact that the analytical SERS EF are the same for both dyes under a given set of experimental conditions. This agreement, for example, automatically excludes potential problems of different adsorption properties. We believe these results indicate that the enhancement is mainly electromagnetic in nature here (unless both molecules happen to have accidentally the same chemical enhancement, which is an unlikely situation). Similar evidence for CV and RH6G has been found before.[5]

In more general terms, our paper here shows a quantitative application of SERS, that allows the experimental estimation of an extremely difficult intrinsic physical property of molecules (the resonant differential Raman cross section). Arguably, one of the main point of criticism of SERS over the years has been its inability to produce reliable quantitative results, which is a trend that is being slowly reverted in recent times by the development of better and more reproducible SERS substrates. Our method here "circumvents" the variabilities in the exact

enhancement factor by maximizing as much as possible spatial and time averaging of the signals and by using two dyes simultaneously. It also highlights one of the less commonly utilized benefits of SERS (as opposed to the SERS enhancements), namely its ability to quench fluorescence with respect to Raman.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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S.I.   SYSTEM RESPONSE AND REFERENCE CV CROSS SECTIONS

For crystal violet (CV) we measured the resonant Raman cross section (Fig. 3 of the main paper) of the $1620\,\mathrm{cm}^{-1}$ mode by comparison with a standard: 2-bromo-2-methylpropane (2B2MP) (thoroughly characterized in Ref. 1). We use, in particular, the $516\,\mathrm{cm}^{-1}$ Raman mode of 2B2MP which has a differential cross section of $5.4 \times 10^{-30}\,\mathrm{cm}^2/\mathrm{sr}$ at $633\,\mathrm{nm}$ and can be corrected for other excitation wavelengths λ_{ex} by the factor $(633/\lambda_{\mathrm{ex}})^4$ where λ_{ex} is in nm (since it is far from resonance across the visible). By knowing the concentration of CV in a reference solution and the density of 2B2MP, a direct intensity ratio provides the differential cross sections for the former, even without characterizing the scattering volume [1]. Examples of Raman spectra are shown in Fig. S1 while the experimental conditions used are summarized in Table S.I.
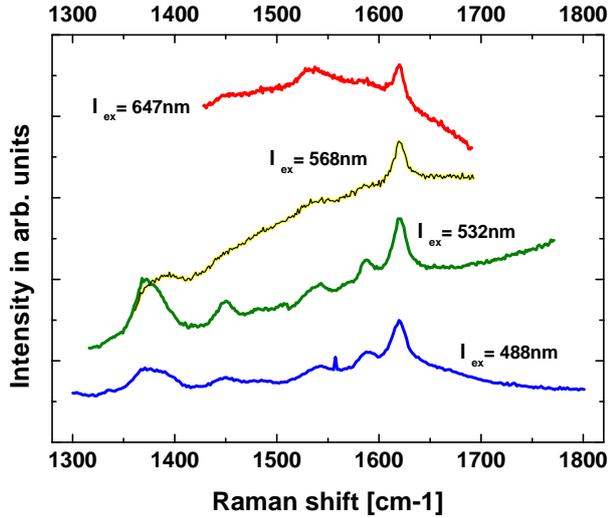

FIG. S1: Examples of bare CV Raman spectra taken at 4 different excitation wavelengths λ_{ex}; all of them show the distinct peak at $1620\ \mathrm{cm}^{-1}$ above a residual fluorescence background.

As mentioned in the main paper, these Raman spectra were taken with a polarizer for detection parallel to the incident polarization. We therefore measure the ratio of $d\sigma_\parallel/d\Omega$ rather than that of $d\sigma/d\Omega$. This can be corrected using the expression:

$$d\sigma/d\Omega = (1 + \rho)d\sigma_\parallel/d\Omega, \qquad (S1)$$

where ρ is the depolarization ratio of the mode [2]. To apply this correction, we have used the value $\rho = 0.18$

TABLE S.I: Experimental conditions (CV concentration c, integration time t, and incident power P), correction factors (for absorption $K_{\mathrm{abs.}}$ and system response $K_{\mathrm{sys.}}$) and measured CV depolarization ratios $\rho = I_\perp/I_\parallel$ used for determining the bare CV cross-sections.

λ_{ex}	458nm	488nm	514nm	532nm	568nm	633nm	647nm
$c\ [\mu\mathrm{M}]$	50	10	10	10	10	100	100
$t\ [\mathrm{sec}]$	25	100	200	100	30	60	100
$P\ [\mathrm{mW}]$	4.0	2.0	2.0	2.0	2.0	2.0	2.0
$K_{\mathrm{sys.}}$	2.74	1.37	1.07	1.02	0.88	0.69	0.62
$K_{\mathrm{abs.}}$	2.44	1.63	2.13	2.84	1.97	2.51	1.43
ρ	0.77	0.29	0.31	0.45	0.36	0.35	0.28

for 2B2MP ([1]) and have measured ρ for CV at all excitation wavelengths. These are summarized in Table S.I. It is interesting to note the variations of ρ as a function of excitation wavelength, a clear indication of mode symmetry changes induced by resonance [3].

There are three possible additional complications to this standard measurement in the case of resonant dyes.

- Firstly, the solution is not transparent, and its absorption of both the laser and Raman light must be taken into account. To correct this, we measured the dye's absorbance and calculated the loss in incident power as well as the loss in scattered light taking into account the shift in wavelength. This leads to a correction factor $K_{\mathrm{abs.}}$ (for each excitation wavelength), which is listed in Table S.I (the focusing distance of the objective is $3.3\,\mathrm{mm}$).

- Secondly, photobleaching could affect the measurement at high power densities. To minimize this, low-magnification ($\times 20$ immersion) and relatively low laser powers were used. The absence of photobleaching was further checked by measuring the signals at two laser powers.

- Thirdly, for a given excitation wavelength λ_{ex} the detection efficiency of the system varies from the reference mode of 2B2MP (@ $516\,\mathrm{cm}^{-1}$) to that of CV (@ $1620\,\mathrm{cm}^{-1}$). To correct this we measured the response of our system (depicted in Fig. S2) by comparing the signal with the black body spectrum of a calibrated lamp of known effective temperature ($T = 2700\,\mathrm{K}$). From this curve the correction factor $K_{\mathrm{sys.}}$ is extracted which is again listed
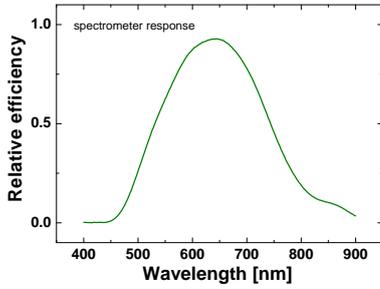
FIG. S2: Response (relative efficiency) of our spectrometer at different wavelengths.

in Table S.I. CV cross-section values in the green-yellow region are not affected much by the different response, but in the blue-purple region the correction can be important. For $\lambda_{\mathrm{ex}} = 458\,\mathrm{nm}$, for example, the $1620\,\mathrm{cm}^{-1}$ mode of CV (at $495\,\mathrm{nm}$) is overestimated by a factor of 2.7 with respect to the $516\,\mathrm{cm}^{-1}$ mode of 2B2MP (at $469\,\mathrm{nm}$) due to the system response and is corrected by $K_{\mathrm{sys.}}$.

S.II. FURTHER DETAILS ON SAMPLES AND PROCEDURES

Figure S3 shows a few examples of measurements done in the Ag Lee & Meisel colloids at three different excitation wavelengths. We take normally several spectra (as shown in the figure) over time (with 30 to 200 sec integration time) to obtain an average. This is done to ensure that the average signal was not affected by an unusually large fluctuation of the signal (which can happen sometimes with large clusters in colloidal liquids). RH6G goes from dominating the spectrum over CV in the green (532 nm) to almost disappearing from it in the red (633 nm). The latter suggests that its cross-section in the red is at least an order of magnitude smaller than CV. In order to verify this, a new sample was prepared where RH6G and CV were diluted as before, but this time with a relative concentration of 10:1 (10 nM in total). Figure S4 shows complementary data (equivalent to the data in Fig. S3) for this additional sample. For the seven wavelengths used for this experiment laser powers between 100 and 1000 μW with the $\times20$ immersion objective were chosen. No detectable effect of photobleaching was observed at this laser power level.

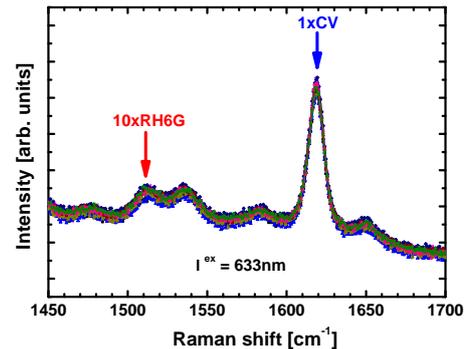FIG. S3: Examples of data for a sample with a concentration ratio RH6G:CV = 1:1 (10 nM in total), measured at different excitation wavelengths: (a) $\lambda_{\mathrm{ex}} = 633\,\mathrm{nm}$, (b) 568 nm and (c) 532 nm.

FIG. S4: Sample with RH6G:CV = 10:1, measured at an excitation wavelength of $\lambda_{\mathrm{ex}} = 633\,nm$. The difference in cross sections is partially compensated by an increase in the relative concentration of the two dyes.

It is worth pointing out at this point that the accu-

rate preparation of low dye concentrations is a challenging aspect of this experiment and should always be done with care (with an external control like fluorescence when possible). As a way of example, with a fluorometer we were able to check concentrations down to ~1 nM and observed deviations from the desired value of up to a factor

of ~ 3 in many instances. With the final procedure we followed (which included carefully electrostatically discharging the equipment we used) we were able to follow a reliable dilution allowing us to obtain the desired concentrations with an accuracy of about ~ 10% in the final concentration.

S.III.   ANALYSIS OF BI-ANALYTE SPECTRA

In this section we outline the procedure we used to obtain the differential SERS cross-section ratios for the different excitation wavelengths λ_{ex}:

$$R(\lambda_{\mathrm{ex}}) := \sigma_{\mathrm{RH6G}}^{1510}(\lambda_{\mathrm{ex}}) \, / \, \sigma_{\mathrm{CV}}^{1620}(\lambda_{\mathrm{ex}}) \qquad \text{(S2)}$$

Assuming that both RH6G and CV experience the same enhancement factor in a bi-analyte SERS sample, this will also correspond to the ratio of differential Raman cross-section (see main text for discussion).
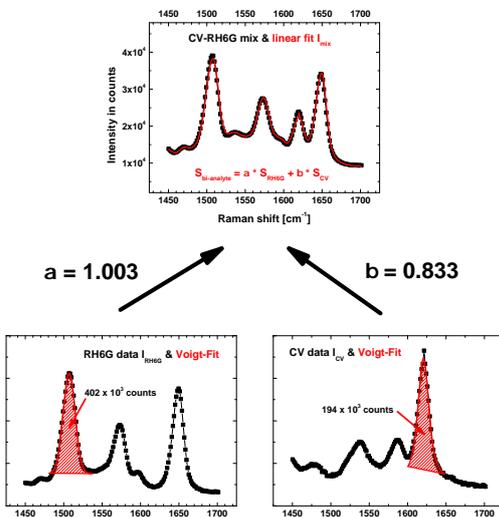

FIG. S5: Schematic illustrating the method to extract the ratio R of relative peak intensities from a bi-analyte spectrum (top spectrum). First the peak intensities $\Sigma_{\mathrm{RH6G}}^{1510}$ and $\Sigma_{\mathrm{CV}}^{1620}$ of a pure RH6G (S_{RH6G}, bottom left) and a pure CV SERS spectrum (S_{CV}, bottom right) are determined. In a second step a linear least-square decomposition of the mixed spectrum S_{mix} into its components S_{RH6G} and S_{CV} (and a quadratic background) yields factors α and β. Note the accuracy of this fit. The ratio is then simply obtained from Eq. S5.

It is obvious that R is equal to the ratio of the integrated peak intensities Σ of the respective dye in the mixed sample:

$$R = \Sigma_{\mathrm{bi-analyte}}^{1510}/\Sigma_{\mathrm{bi-analyte}}^{1620} \qquad \text{(S3)}$$

However extracting those peak intensities from the bi-analyte SERS spectra is not trivial. The problem we are facing is possible overlap of peaks belonging to the different dyes which makes the fitting in the bi-analyte spectrum prone to error.

To avoid this potential overlap problem, for each excitation wavelength λ_{ex}, the fits of the peaks were first performed on a pure RH6G and on a pure CV SERS spectrum (S_{RH6G} and S_{CV} respectively). This provides us with peak Intensities $\Sigma_{\mathrm{RH6G}}^{1510}$ and $\Sigma_{\mathrm{CV}}^{1620}$ for each dye in two reference samples not affected by peak overlap. Those pure SERS spectra were then taken as a basis for a linear least-square decomposition of the bi-analyte data ($S_{\mathrm{bi-analyte}}$) yielding factors α and β that fulfill the following equation:

$$S_{\mathrm{bi-analyte}} = \alpha\, S_{\mathrm{RH6G}} + \beta\, S_{\mathrm{CV}} \; + \text{background} \quad \text{(S4)}$$

The background (which consists of instrumental background and fluorescence) was taken as a quadratic function of the wavelength (which preserves the linearity of the decomposition). This linear least-square decomposition of a mixed SERS spectrum is extremely accurate since it is not affected by non-symmetric peak shapes or peak overlap.

Knowing α and β the ratio R is then easily obtained:

$$R = \left(\alpha\Sigma_{\mathrm{RH6G}}^{1510}\right) / \left(\beta\Sigma_{\mathrm{CV}}^{1620}\right) \qquad \text{(S5)}$$

This procedure is summarized in Fig. S5.

[1] E. C. Le Ru, E. Blackie, M. Meyer, and P. G. Etchegoin, J. Chem. Phys. C **111**, 13794 (2007).

[2] E. C. Le Ru and P. G. Etchegoin, *Principles of Surface Enhanced Raman Spectroscopy and Related Plasmonic Effects* (Elsevier, Amsterdam, 2009).

[3] S. Buchanan, E. C. Le Ru, and P. G. Etchegoin, Phys. Chem. Chem. Phys. **11**, 7406 (2009).