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# Specificity and Sensitivity of Fluorescence Labeling of Surface Species

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FLOSS (fluorescence labeling of surface species) enables one to identify and quantify very low concentrations of surface functional groups. Unlike most surface analytical techniques, FLOSS can provide absolute, as well as relative, surface coverage determination. However, as with any other surface derivatization technique, FLOSS provides a lower limit to surface coverage. The specificity of FLOSS for a particular functional group is the key to this application. In one FLOSS protocol, amine-modified dyes are used to label surface aldehyde groups. However, amine-modified dyes, in principle, can bind to both aldehyde and carboxyl groups, limiting specificity. In this paper, we report that the FLOSS protocol devised results in less than 0.5 % of the carboxyl-modified dyes binding to the surface amine groups. Therefore, the presence of carboxyl groups on the surface should have a limited effect on the detection of aldehyde groups by amine-modified dye. Quenching of fluorescence can potentially affect quantitative measurements. To address this issue, the densities of surface functional groups of CHO-, $NH_2$-, and epoxy-coated glass surfaces were quantified using FLOSS and compared to surface densities estimated by other methods. The FLOSS technique was extended to glass surfaces by using visible absorbing and emitting dyes. The lower detection limit is on the order of $10^9$groups/cm$^2$.

## Introduction

Precise identification and quantification of functional groups on surfaces is not an easy task, especially for surfaces with very low functional group densities.[1−10] However, the nature and density of surface functional groups are essential characteristics that affect the properties and applications of the surfaces, for example, in surface wetting,[11,12] protein adsorption,[13] cell adsorption,[14] and biosensors.[15] Understanding the surface chemical composition is of great interest for these applications.

Determination of surface functional group densities is a difficult problem in surface science. The sensitivity of common surface techniques is limited. X-ray photoelectron spectroscopy (XPS) and infrared spectroscopy (IR) have difficulty detecting surface groups below $10^{12}$ groups/cm$^2$.[16] Time-of-flight secondary ion mass spectrometry (ToF−SIMS) is a sensitive surface characterization technique, but the application of ToF−SIMS for the detection of surface functional groups has a reported detection limit that is on the order of $10^{13}$ molecules/cm$^2$.[5,7] In addition, the ease of operation is also important for performing surface analysis. Some surface-sensitive techniques, e.g. XPS and SIMS, need to be operated in ultrahigh vacuum (UHV) environment and the sample may be destroyed as a result of the measurement. This is even more critical when analyzing biological samples.

What is actually more difficult is to determine the absolute value of surface functional group densities. Common techniques are good at measuring relative surface concentrations. However, translating the signal detected to an absolute value of functional groups is nontrivial.

Fluorescence labeling is a promising method because of its high sensitivity, ease of operation, and in situ applicability. It has been widely used in biological applications,[17−22] polymer chemistry,[2,3,23−26] and the study of self-assembled monolayers.[4,27−30] Similarly, electron spin resonance (ESR) of chemically bound

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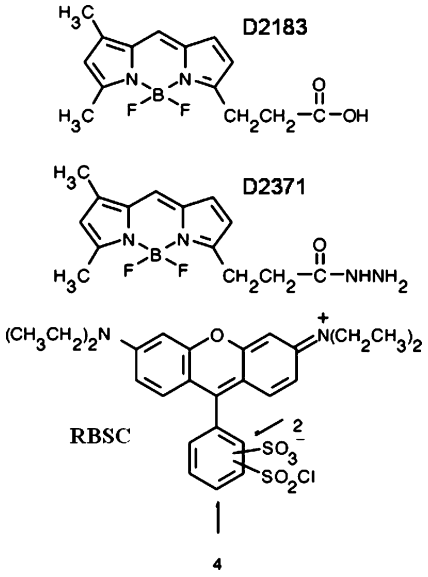
**Scheme 1. Structures of Dyes Used in This Paper**




spin-labels has been applied to the quantitative study of functional group density.[31−33] To achieve precise identification and absolute quantification of surface functional groups, FLOSS (fluorescence labeling of surface species), a procedure for labeling, fluorescence detection, and calibration, was proposed.[6] The lower detection limit was suggested to be $\sim 10^{11}$ molecules/cm$^2$, below the detection limit of common surface techniques, e.g., XPS and IR.[16] FLOSS is in principle nondestructive. By choosing a reversible labeling reaction, the initial surface can be recovered after FLOSS detection, if required. Moreover, FLOSS does not require the UHV environment necessary for XPS and SIMS.

The specificity of the labeling reactions is the key issue that determines the applicability of FLOSS. Of the three labeling schemes used previously,[6,8] the specificity of reaction of aldehyde groups with amine groups is perhaps the most questionable. Carboxyl groups can also bind with amine groups to form ionic bonds at room temperature.[34] It is also possible for the ionic bonds to be dehydrolyzed and form amide bonds, as in the synthesis of nylon 66.[35] (The formation of amide bonds requires either a catalyst, e.g. carbodiimide, or high pressures and temperature.) However, as carboxyl groups and aldehyde groups may coexist on a surface,[6] it is important to investigate the influence of carboxyl groups on the detection of aldehyde groups with amine-modified dyes.

Glass surfaces are used in many applications, e.g., biological microarrays. Fluorescent dyes that absorb and emit UV light are not suitable for FLOSS on glass, since this material absorbs UV light, leading to a strong fluorescence background. To expand the FLOSS technique to biological applications, the fluorescent dyes should absorb, and fluoresce, in the visible region.

## Experimental Section

**Reagents.** All fluorescent dyes (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid, catalog no. D2183; 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid, hydrazide, catalog no. D2371; and Lissamine rhodamine B sulfonyl chloride (RBSC), catalog no. L1908) were purchased from Molecular Probes (Invitrogen) (Scheme 1).

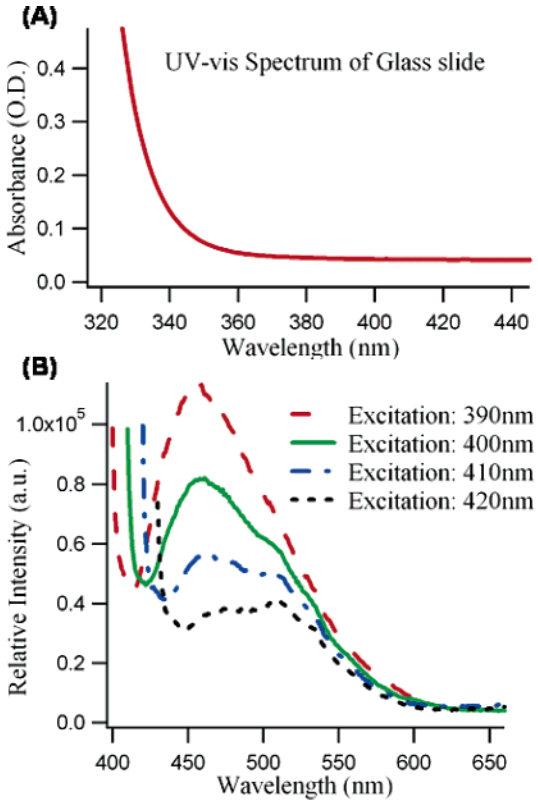



**Figure 1.** (A) UV−vis spectrum of a glass slide (SCHOTT). (B) Fluorescence emission spectra of SCHOTT glass slide as a function of excitation wavelength.

**Substrates.** All coated glass slides (amine-coated slide A; aldehyde-coated slide AL; and epoxy-coated slide E) were provided by SCHOTT Nexterion. The slides are coated via a dip-coating process. The low self-fluorescence glass slides first undergo a rigorous cleaning cycle to make sure that the surface is free from particulate matter and also cleaned of any adventitious carbon. The coatings are deposited from organic solvents using standard liquid-phase silanization procedures.[36]

**Fluorescence Labeling Reaction Condition.** All labeling reactions are performed by putting the coated glass slides in 10 $\mu$M methanol solutions of chromophore at room temperature for 2 h.

**Postreaction Cleaning Procedure.** Following the labeling reaction, all samples were rinsed twice with neat methanol and then sonicated successively in less polar solutions ($CH_2Cl_2$, acetone, and hexane) for 10 min, respectively.

**Fluorescence Measurements.** All the fluorescence measurements were performed on a HORIBA Jobin Yvon Spex Fluorolog 3 fluorimeter with 5-nm band-pass and three-scan averages. All samples were oriented at a 45° angle with respect to the excitation beam with a front-face setup for detection. All spectra were normalized by a source reference photodiode to correct for lamp intensity fluctuations.

**UV−Vis Measurements.** A Perkin-Elmer Lambda 19 UV−vis spectrometer with a 1-mm cell was used for UV−vis experiments.

**Calibration Curves.** All calibration curves were made using a drop and dry method. Five-microliter drops of diluted (1−100 nM) chromophore solutions were placed on the glass surface, on which a rectangle (4 mm × 6 mm) was drawn by a diamond scribe to confine the solution. Fluorescence measurements were made after each drop.

**Hydrolysis of Imine.** To break the imine linkage, between the amine-modified dye (D2371) and the aldehyde-coated slide, the

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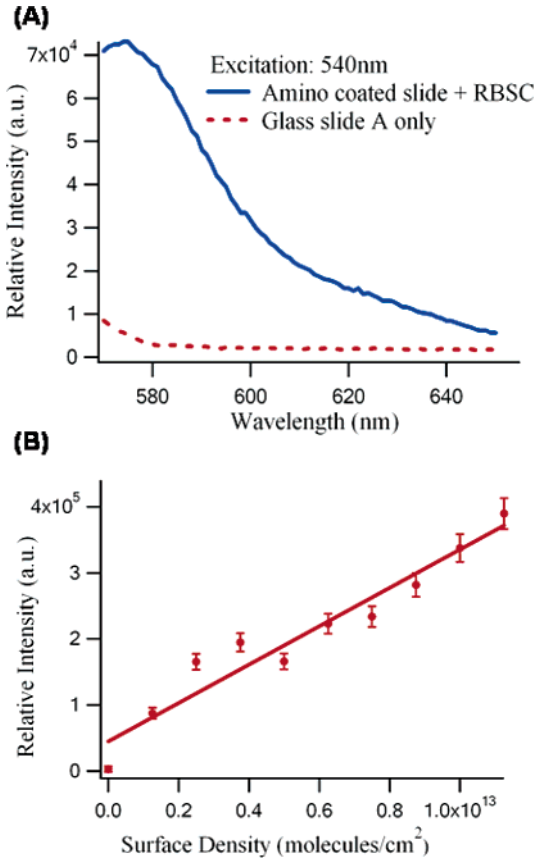


**Figure 2.** (A) Fluorescence detection of RBSC-reacted glass slide A (amine-coated) and intrinsic fluorescence of glass slide A (dashed line). (B) Calibration curve for fluorescence at 580 nm from RBSC on glass slide A.

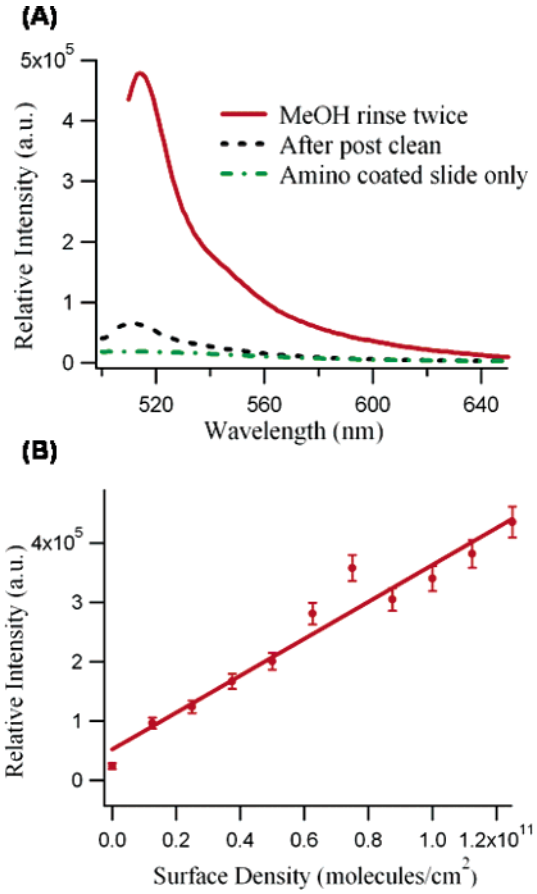


**Figure 3.** (A) Fluorescence detection of two D2183-reacted amine-coated slides. One sample is only rinsed in MeOH twice after the reaction (red solid line), the other sample is subjected to the full postreaction cleaning procedure (black dash line). The dashed−dotted line is the intrinsic fluorescence of the clean amine-coated slides. (B) Calibration curve for dye D2183 (Scheme 1). All samples were excited at 470 nm.

labeled the slide was soaked in methanol containing 0.1 M hydrochloric acid for 1 h at room temperature. The sample was then sonicated in methanol for 5 min. All the solution was recovered for fluorescence detection.

## Results and Discussions

In the original FLOSS paper,[6] all the fluorescent dyes absorb and emit UV light. These dyes cannot be used on glass surfaces, which absorb UV light and fluoresce strongly in the same region as the chromophores originally employed (Figure 1). Therefore, the fluorescent dyes used in the original FLOSS paper[6] are not useful with glass surfaces. To apply FLOSS on glass substrates, the absorption wavelength of labeling dye should be at least 420 nm (Figure 1B) to achieve an acceptable level of background signal. The fluorescent dyes selected (Scheme 1) all absorb and fluoresce in the visible region. Thus, the extension of the FLOSS technique to the visible region was demonstrated. This opens up the possibility that FLOSS can be used in biologically related applications where glass slides are commonly employed.

To determine if binding of carboxyl groups to amine groups might interfere with detection of aldehyde groups, i.e., to quantify the chemical specificity of FLOSS, amine-coated glass slides were reacted with carboxyl-modified dye (D2183, Scheme 1) and fluorescence detection was performed. Before this investigation, the number of amine groups on an amine-coated glass surface was first quantified. The amine groups on the glass slide were labeled with molecular dye RBSC (Scheme 1) and the fluorescence signal was detected.[17] The number of dyes on the slide surface is determined to be $(1.0 \pm 0.3) \times 10^{12}$/cm$^2$, using the RBSC calibration curve. (Figure 2).

After reacting the amine-coated glass slide with carboxyl-modified dye (D2183), fluorescence measurements were performed on two samples, a sample rinsed only by MeOH twice and a sample cleaned with the full postreaction cleaning procedure (Figure 3A). The fluorescence signal from the sample cleaned with the full postreaction cleaning procedure was reduced by 1 order of magnitude, indicating that most of the dye molecules adsorbed on the MeOH rinsed slide surface are physisorbed and could be removed by the full postreaction cleaning procedure.

A calibration curve was made to quantify the number of dye molecules that remained on the slide surface before and after full postreaction cleaning procedure (Figure 3B). According to the calibration curve, the fluorescence intensity of the sample cleaned with the full postreaction cleaning procedure in Figure 3 corresponds to a surface dye molecule density of $(3.5 \pm 2.3) \times 10^9$ per cm$^2$. On the basis of the surface density of amine groups determined above, only 0.36% of the amine groups on the slide surface reacted with the carboxyl-modified dyes. Clearly, at room temperature, carboxyl groups do not strongly bind to surface amine groups. However, the aldehyde groups can covalently bind to amine groups with good efficiency. An aldehyde-coated glass slide was labeled with amine modified dye (D2371). The fluorescence signal collected (Figure 4) corresponds to a surface aldehyde group density of $(4.8 \pm 0.3) \times 10^{12}$ per cm$^2$.

It should be pointed out that the reaction between surface-bound carboxyl groups and solution-phase amine groups may be different from the reaction between surface-bound amine groups

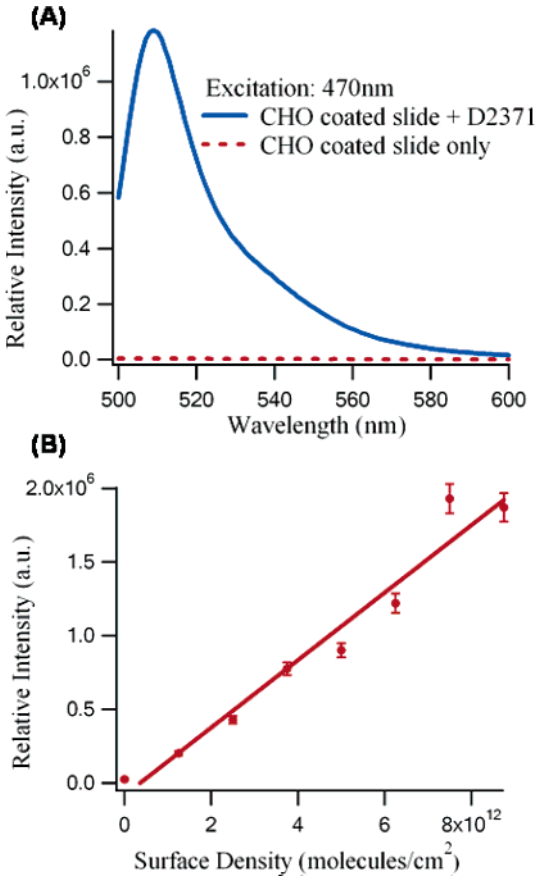


**Figure 4.** (A) Fluorescence detection of D2371-reacted glass slide AL (aldehyde-coated) and unreacted AL glass slide (dashed line). (B) Calibration curve for dye D2371 (Scheme 1), excited at 470 nm.

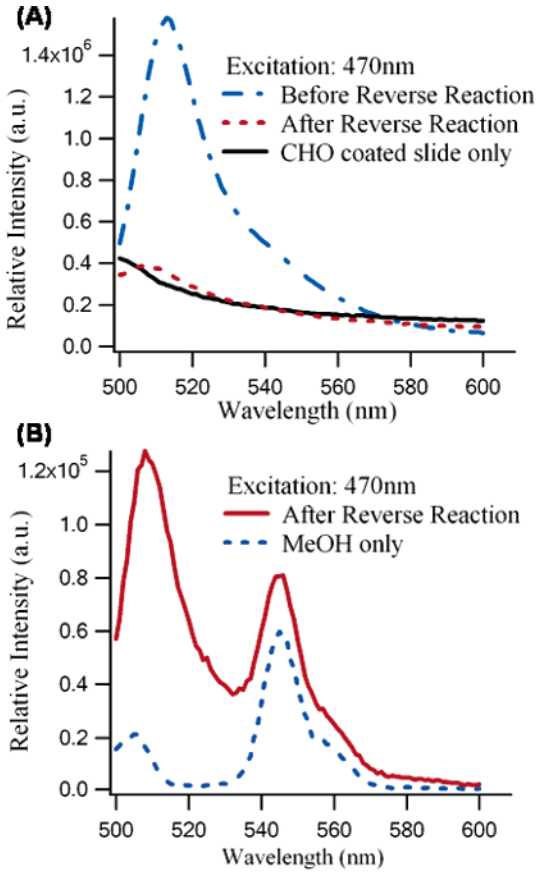


**Figure 5.** (A) Fluorescence detections of D2371-reacted slide AL (aldehyde-coated) before and after reverse reaction. (B) Fluorescence detection of the solution after reverse reaction.

and solution-phase carboxyl groups. While perhaps less than ideal, we chose the latter reaction due to the lack of pure carboxyl-terminated glass substrates. To our knowledge, there is no direct method to grow a self-assembled monolayer of carboxyl groups on glass surfaces. The commonly used methods include the oxidation of terminal double bonds to COOH[37,38] and the hydrolysis of esters[39,40] or succinic anhydrides.[41,42] However, there is no guarantee that the oxidation or hydrolysis reactions are complete. Hence, unexpected surface functional groups (starting material or intermediate) could appear on the surface. The presence of other functional groups on the COOH surface, especially amine-reactive aldehyde groups and succinic anhydride groups, would make the determination of binding efficiency between carboxyl groups and amine groups questionable. The study of the efficiencies of end-group-converting reactions, while of significant interest, is beyond the scope of this paper. In our study, the amine groups are bound to the surface through an alkane chain, spacing the amine groups away from the surface.[36] The carboxyl group is also linked to the dye via an alkyl chain. Therefore, while we cannot rule it out, we expect that, under the present circumstance, surface enhancement or inhibition of the labeling reactions is limited. This may be more critical when the functional groups are not attached to flexible chains.

To confirm the accuracy of the surface fluorescence detection and calibration procedures, a different method was used to quantify the number of dye molecules bound, and by inference the amine group density, on the surface of the aldehyde-coated slide. After the slide was labeled with the amine-modified dye (D2371, Scheme 1), it was subjected to hydrolysis for 1 h by 0.1 M HCl to cleave the imine bond. The emission signal from the glass slide was reduced to very close to the background level (Figure 5A), indicating that most of the imine bonds were cleaved and the dye molecules were removed from the surface to the solution. The concentration of dye D2371, released in the 20 mL solution after the reverse reaction, is determined from solution fluorescence to be $1.5 \pm 0.05$ nM, corresponding to an initial surface bound density of $(7.1 \pm 0.2) \times 10^{12}$ D2371 molecules per $cm^2$.(Figure 5B). The surface fluorescence detection result is $(4.8 \pm 0.3) \times 10^{12}$ per $cm^2$ (Figure 4). This result suggests some fluorescence quenching due either to the surface or to chemical bond formation with the surface. Nevertheless, FLOSS detects a surface functional group density that is similar to that determined by solution detection. In addition, FLOSS is much easier to perform. It should be noted that the solution detection depends on the reversibility of the labeling reaction, while FLOSS does not. However, we acknowledge that the FLOSS technique probably provides a lower limit to the number of surface functional groups.

In the original FLOSS paper, using UV absorbing and emitting species, the lower detection limit was determined to be $10^{11}$ molecules/$cm^2$.[6] In this paper, the lower detection limit appears to be at least 2 orders of magnitude smaller. In the surface

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**Table 1. Comparison of Fluorescence and UV−Vis Detection**

| slide | dye used | FLOSS result (molecules/cm$^2$) | UV−vis result (molecules/cm$^2$) |
|---|---|---|---|
| A ($NH_2$ coating) | L1908 (sulfonyl chloride) | $(1.0 \pm 0.3) \times 10^{12}$ | $<8.4 \times 10^{11}$ [a] |
| E (epoxy coating) | D2371 ($NH_2$ modified) | $(5.6 \pm 0.3) \times 10^{12}$ | $3.7 \times 10^{12}$ |
| AL (CHO coating) | D2371 ($NH_2$ modified) | $(4.8 \pm 0.3) \times 10^{12}$ | $2.7 \times 10^{12}$ |

[a] Under detection limit.

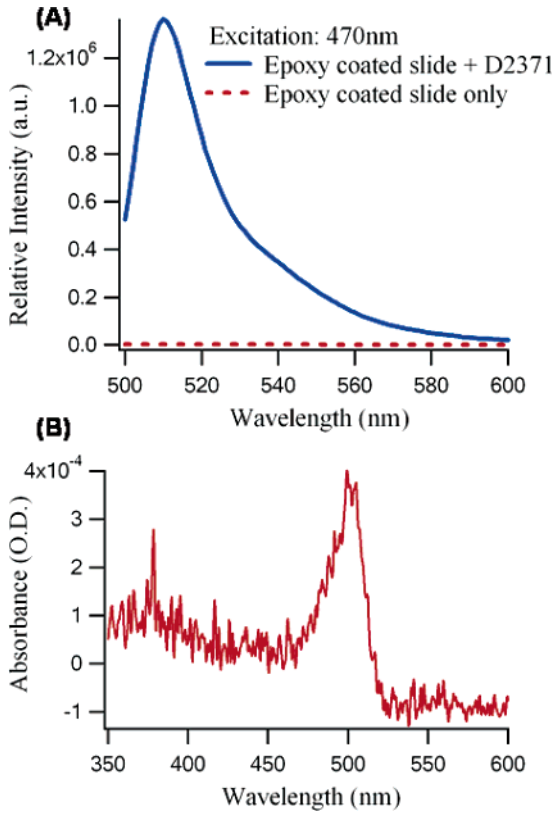


**Figure 6.** Fluorescence detection (A) and UV−vis detection (B) of D2371-reacted glass slide E (epoxy-coated).

fluorescence detection experiments presented here, for example Figure 3, the background is on the order of $10^3$ cps, and it could probably be reduced further. On the basis of that background level, a $10^4$ cps signal can be easily detected, which corresponds to a surface functional group density of $\sim 10^9$/cm$^2$.

The upper limit of FLOSS depends on the size of the labeling dye molecule. To well separate the labeling dye molecules on the surface, given the van der Waals radii of the dyes, the surface functional group density should be lower than one group per 2 nm × 2 nm, which is $2.5 \times 10^{13}$ per cm$^2$. While FLOSS is a sensitive technique, it is not ideal for detecting high concentrations of surface functional groups, limiting some applications of FLOSS. However, for these situations a number of techniques already exist.

Since glass slides are transparent in the visible region, UV−vis absorbance can also be used to quantify the density of chromophores on the surfaces after labeling. The 0.4 mOD peak at 490 nm (Figure 6b) for an epoxy-coated glass slide (slide E) reacted with dye D2371 corresponds to a surface functional group density of $3.7 \times 10^{12}$/cm$^2$, taking into account that both sides are labeled with dye. The density is comparable to the findings of the FLOSS experiments, shown in Figure 6a, in which the peak corresponds to $(5.6 \pm 0.3) \times 10^{12}$ molecules/cm$^2$ on the surface. We performed both UV−vis and fluorescence detection on glass slide A, slide AL, and slide E (Table 1). The detected surface functional group densities from UV−vis detection and fluorescence detection are similar, indicating that there is no significant surface fluorescence quenching. The comparison also highlights how much lower the detection limit of fluorescence detection is compared to UV−vis. It is difficult for UV−vis to detect signals smaller than 0.1 mOD since the noise is on the order of 0.1 mOD. Therefore, the lower detection limit of UV−vis detection will be no lower than $10^{11}$molecules/cm$^2$ with the dyes employed in this study.

As a sensitive surface characterization technique, FLOSS can be applied to a number of areas. However, the methods used to validate the results of FLOSS, e.g. the reverse reaction method, and the UV−vis absorbance method, all rely on the efficiency of labeling reactions. While we would like to use additional validation methods, the problem is that many other techniques do not have the sensitivity to work at the detection range of FLOSS.

## Conclusions

Under the conditions employed for FLOSS experiments, carboxyl groups do not bind strongly to amine groups on a surface. The postreaction cleaning procedure employed for the FLOSS experiments effectively removes weakly bound dye molecules (physisorbed) and dyes bound by ionic bonds. With these precautions, the labeling of aldehyde groups by amine-modified dye will not be affected by the presence of carboxyl groups, confirming the specificity of FLOSS for aldehyde and amine group detection. By choosing appropriate dyes, FLOSS can be applied to glass surfaces, extending the application of FLOSS. The detection limit of FLOSS with the visible absorbing and emitting dyes employed in this study is on the order of $10^9$ molecules/cm$^2$, while most of the other commonly used surface-sensitive techniques have trouble detecting a surface functional group density lower than $10^{12}$/cm$^2$. However, our results suggest that fluorescence quenching needs to be considered in absolute quantitative measurements, though it does not appear to affect relative measurements.

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