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Double interference fluorescence enhancement from reflective slides: Application to bicolor microarrays

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A technique for enhancing fluorescence from species close to a substrate is described, based on a multilayer dielectric mirror coating. For proper design, interferences bring improvements to both fluorescence excitation and collection, each about four fold. The overall improvement reaches 10 to 15 fold as compared to a standard glass slide. We apply this to so-called deoxyribonucleic acid (DNA) chips, that is to DNA hybridization assays on microarray platforms, for which two fluorophores most commonly used are cyanine dyes (Cy3™ and Cy5™). Impact of the two-color scheme is discussed. A validation method based on spin-coated fluorescent ultrathin layers is shown to accurately determine the amplification factors of the different tagging dyes. © 2005 American Institute of Physics. [DOI: 10.1063/1.1999018]

A large detection sensitivity is extremely important in assays for analysis of gene expression patterns, patient genotypes, drug metabolism, etc., and especially when ribonucleic acid or complementary deoxyribonucleic acid is involved. While many detection schemes exist (based on ellipsometry,6 surface plasmon resonance,3–4 fluorescence excitation and collection, each about four fold. The overall improvement reaches 10 to 15 fold as compared to a standard glass slide. We apply this to so-called deoxyribonucleic acid (DNA) chips, that is to DNA hybridization assays on microarray platforms, for which two fluorophores most commonly used are cyanine dyes (Cy3™ and Cy5™). Impact of the two-color scheme is discussed. A validation method based on spin-coated fluorescent ultrathin layers is shown to accurately determine the amplification factors of the different tagging dyes. © 2005 American Institute of Physics. [DOI: 10.1063/1.1999018]

processes. To simplify, FC and FE are first taken as monochromatic and the mirror is represented for physical purpose as a transparent layer of index similar to the upper medium onto a perfect metallic reflective plane. This reflection has an amplitude |r(λ)| and a dephasing φ = ±π. The location of fluorophores is assumed to be less than λ/20 above the top surface.

Let θex be the FE direction and θflu be one direction in the FC solid angle. Due to interference effects, the exciting field intensity at the surface, referenced to a vacuum, is of the form

![Figure 1](image_url)
The phase $\varphi_{exc}$ now depends on $\lambda$ and $\theta_{exc}$ [see Ref. 7, $\varphi_{exc}=2\pi \delta(0) \cos(\theta_{exc})/\lambda$, $\delta(0)$ being the normal incidence roundtrip]. These interferences could be constructive for

$$r = -1 \quad \text{and} \quad \varphi_{exc} = (2p + 1) \pi.$$  

Ensuring the presence of a field antinode of the excitation light at the upper face of the mirror. This straightforward amplification can be doubled up by a distinct enhancement of the emission light, also based on interference effects for emission from a dipole. Actually, for FC, a similar formula holds: In the far field, the direct and reflected fields add up, leading to

$$G_{flu} = |1 + r(\lambda, \theta) \exp[i \varphi_{flu}]/(\lambda, \theta)|^2. \quad (3)$$

Thus, for the ideal case $r = -1 = e^{i\pi}$, optimal enhancements are obtained for constructing interference, that is for Eq. (2) or $\varphi_{flu} = (2p' + 1) \pi$ ($p, p'$ integers): both FE and FC processes are then enhanced by a factor of $G_{exc} = G_{flu} = 4$ with respect to a vacuum, leading to an overall enhancement $G = G_{exc} \times G_{flu} = 16$. Integers $p'$ and $p$ may differ.

Note that if the ratio $\delta(0)/\lambda$ is much larger than unity (case of a far mirror), phase relations are lost within minute angular or spectral shifts and signal enhancements $G_{exc}$ and $G_{flu}$ average to 2, hence $G = 4$ only.

Turning to real systems such as the Bragg mirror illustrated in the inset of Fig. 1(b), main changes arise from the different dependences of the reflection phase $\varphi(\theta, \lambda)$ and amplitude $r(\theta, \lambda)$. In addition, a natural “unamplified” reference is a glass surface, not a vacuum: for glass, $r(\theta=0^\circ)= -0.18$ and $\varphi \approx 0$, leading to an inhibition along the glass normal, $G_{glass} = 0.82^2 = 0.67$ or less. Some poor fluorescence results for silicon wafer substrates in the literature just have, we believe, this origin: With a larger $|r| \approx 0.5$, one has $G < 0.1$ for a thin native silica layer (a few nm), a clear illustration of how drastic these effects can be.

In practice, enhancement is sought for two fluorophores, e.g., Cy3TM ($\lambda_{exc} = 532$ nm, $\lambda_{flu} = 570$ nm) and Cy5TM [$\lambda_{exc} = 633$ nm, $\lambda_{flu} = 670$ nm, see Fig.1(c)], with some FE linewidth and for some FC aperture the excitation beam being collimated. The issue is therefore how much phases $\varphi$ move away from $(2p + 1) \pi$ for this extended set of conditions. We focus in the following on designs where the above phases vary as little as possible ($p \neq p'$, Cy3TM and Cy5TM). Within this option, one still has to select either a metallic mirror, with a slowly varying phase $\varphi(\theta, \lambda)$, or a dielectric mirror with a more rapidly varying phase. Reflectivities of silver and aluminum are high so that such a metal with a thin SiO2 layer actually yields a very good gain $G$, for a typical 100 nm SiO2 thickness (quarter wave). However, due to the poor suitability of Ag or Al to various chemical protocols, dielectric mirrors are preferred. A slowly varying phase arises in the stopband of a Bragg mirror (alternate low/high index stack, with indices $n_H$, $n_L$), the slope decreasing for increasing index contrast $n_H/n_L$. Hence, we select the TiO2/SiO2 material pair. In this case, the stopband accommodates the four wavelengths of interest. We validate this approach in the following.

Figure 1(b) represents the reflection phase $\varphi_{exc}$ for a four period TiO2/SiO2 stack on glass (inset, quarter-wave optical layers at the Bragg wavelength $\lambda_B = 612$ nm) at normal incidence and 45° incidence [transverse electric (TE) polarization]. Compared to the somewhat broad FE of bare Cy3 and Cy5 [Fig. 1(c)], filters in existing scanners reduce the relevant width to about $\Delta \lambda = 40$ nm. Then, between 530 and 740 nm, a linear phase is a fair approximation. In other words, the behavior of Bragg mirrors can be approximated to the simpler case of Fig. 1(a) in the stopband, provided the adequate penetration depths are introduced. For large index contrasts, one should carefully distinguish the spectral penetration depth (related to $\partial \varphi/\partial(\lambda)$) from the angular depth [related to $\partial \varphi/\partial \cos(\theta)$] to get the main trends of $G$ in the actual conditions. We rather present here $G$ data [Eqs. (1) and (2)] based on exact calculations, and in the $\varphi_{exc} = \theta_{flu}$ $= 0^\circ$ case for simplicity. The predicted single-wavelength normal incidence enhancements are shown in Fig. 2, while the averages for the FE spectra of Fig. 1(c) and the FE collection numerical aperture (NA) = 0.6 (TE polarization) are shown as a dotted line.

In the experiment, we used microscope glass slides coated by industrial suppliers of optical coatings producing compact TiO2/SiO2 layers. We performed standard hybridization assays on either a commercial glass microarray slide or a mirror coated slide; quantities of spotted and tagged DNA, in the micromolar range, are those typical of such
changes only to second order, so that the stopband position is the main issue. Given the constant slope of Fig. 1(b), a change in stopband position translates into a horizontal shift of the curve of Fig. 1(b). Because, in order to compromise the two fluorophore performances (Fig. 2), none of the G’s is at an extremum, the differential impact on G’s is quite sizable, even for shifts $\Delta \lambda_B$ of only a few nm. An important imbalance $\Delta G$ between the two fluorophores gains can further bias biological interpretation if no care is taken.

For a batch we investigated, we found out through in-depth analysis of transmission-reflection data that the Bragg wavelength $\lambda_B$ was monitored to 1–2 nm accuracy through the position $\lambda_{\text{blue}}$ of the secondary stopband peak [inset of Fig. 1(b)], a study not detailed here. The data points in Fig. 4 refer to this batch. Figure 4 also shows the calculated evolutions of $G_1$ (solid curve) and $G_3$ (dotted curve) as a function of Bragg mirror wavelength ($\lambda_B$), again taking into account the spectral width and NA=0.6.

It is seen that a good agreement is obtained. This result shows that careful validation is necessary to establish the enhancements G brought by mirror slides, especially the balance between the two fluorophores. This is of practical importance since this ratio is the basic data used for further biological analysis, in particular in differential genetic expression assays. The ultrathin dye-doped layer method is a proper assessment of fluorescence enhancement of reflective slides, comparing favorably with the more indirect analysis of reflection-transmission data.

In conclusion, a large enhancement of fluorescence of immobilized species was shown based on properly designed dielectric coated slides that may serve directly as substrates for microarrays. A slide coated with a four-period Bragg mirror, centered around 612 nm, yielded a 10–15 fold enhancement compared to a glass slide. We will detail elsewhere the signal-to-noise ratio enhancement relevant to genetic, biological, or biochemical studies. A physical validation based on fluorescent dye-doped ultrathin layer coating, was described and used to assess gain enhancements and their proper balance between the two fluorophores.