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

Houtai Choumane, Khoi-Nguyen Ha, Constantin Nelep, Alain Chardon, Georges-Olivier Reymond, Carole Goutel, Gordana Cerovic, François Vallet, Claude Weisbuch, Henri Benisty

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H. Choumane,[a] N. Ha, C. Nelep, A. Chardon, G. O. Reymond, C. Goutel, G. Cerovic, F. Vallet, and C. Weisbuch
Genewave, bât 404-XTEC, Ecole Polytechnique, 91128 Palaiseau Cedex, France

H. Benisty
Laboratoire Charles Fabry de l'Institut d'Optique, UMR 8501 du CNRS, 91403 Orsay, France

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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 electrochemistry,[1] surface plasmon resonance,[2–4] ellipsometry,[5] etc.), fluorescence is a preferred detection technique on account of its high intrinsic sensitivity, excellent selectivity, and specificity. Fluorescence-based microarrays discussed here enable very compact assays. It involves fluorescent dyes to signal the hybridization between the target and deoxyribonucleic acid (DNA) probes immobilized ("spotted") on the substrate surface at registered locations. The density of fluorophore molecules grafted on DNA strand remains small (1 on oligo-DNA), to limit fluorescence quenching, making the detection of genes with low expression levels, for example, difficult due to the weak signal.

We present in this letter an enhancement technique for fluorescent signals capitalizing on simple interference effects occurring close to a mirror coating the microarray surface underneath the probes. It is also applicable to other luminescence-based assays (chemo and electrochemoluminescence, not discussed here).

Concretely, a typical microarray consists of thousands of spots of known DNA targets immobilized on a microscope glass slide, functionalized with adequate surface attachment chemistry. Dye-tagged DNA targets of interest are hybridized on chip. Two dyes are used, e.g., green cyanine 3 (Cy3™) for the DNA sample and red Cyanine 5 (Cy5™) for a control target DNA serving as a reference to the sample [see Fig. 1(c) for spectra].[6] Excess material, resulting from, e.g., nonspecific adsorption, is washed away before fluorescence mapping takes place in a scanner. The typical sensitivity of existing scanners for a glass slide is in the range of 10 fluorophores per μm^2, the practical resolution being 10 μm. We sketch in Fig. 1(a) the impact of a mirror on the fluorescence excitation (FE) and fluorescence collection (FC) 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 $|r(\lambda)|$ and a dephasing $\varphi=\pi$. The location of fluorophores is assumed to be less than $\lambda/20$ above the top surface.

Let θ_{exc} be the FE direction and θ_{fluo} 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

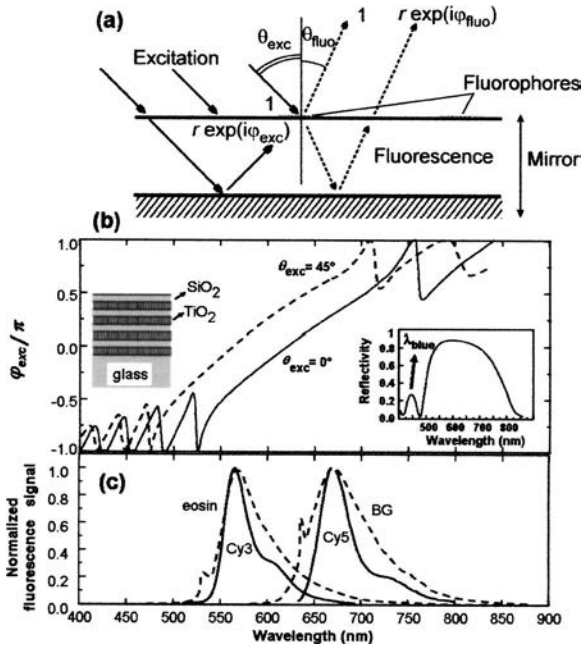

FIG. 1. (a) Fluorescence enhancement with a transparent layer and a metal-like mirror. (b) Reflection phase as a function of wavelength of a TiO_2/SiO_2 Bragg mirror [the inset illustrates a four period Bragg mirror (left) and its reflectivity spectra (right)] for 0 ° (solid line) and 45 ° incidence (dotted line), TE polarization. (c) Fluorescence spectrum of the Cy3™ and Cy5™ markers (solid lines) and of the BG and eosin (dotted lines).

[a]Electronic mail: houtai.choumane@polytechnique.fr

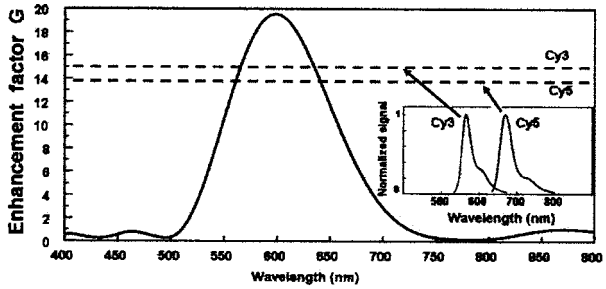

FIG. 2. Enhancement factor at normal incidence for a monochromatic emitter (solid lines) and averages for Cy3™ and Cy5™ fluorophores (dotted lines, spectra in inset) in NA=0.6 aperture (for light collection) and the following excitation wavelengths: $\lambda_{exc}=532$ nm for Cy3™ and $\lambda_{exc}=633$ nm for Cy5™.

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

The phase φ_{exc} now depends on λ and θ_{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

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

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,[8] leading to

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

Thus, for the ideal case $r=-1=e^{i\pi}$, optimal enhancements are obtained for constructing interference, that is for Eq. (2) or $\varphi_{fluo}=(2p'+1)\pi$ (p,p' integers): both FE and FC processes are then enhanced by a factor of $G_{exc}=G_{fluo}=4$ with respect to a vacuum, leading to an overall enhancement $G=G_{exc}\times G_{fluo}=16$. 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_{fluo} 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, $r(\theta=0°)=-0.18$ and $\varphi\sim 0$, 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[9] just have, we believe, this origin: With a larger $|r|\sim 0.5$, 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., Cy3™ ($\lambda_{exc}=532$ nm, $\lambda_{fluo}=570$ nm) and Cy5™ [$\lambda_{exc}=633$ nm, $\lambda_{fluo}=$,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 φ 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 ($p\approx p'$, for Cy3™ and Cy5™). 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 SiO_2 layer actually yields a very good gain G, for a typical 100 nm SiO_2 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 TiO_2/SiO_2 material pair. In this case,[10] the stopband accommodates the four wavelengths of interest. We validate this approach in the following.

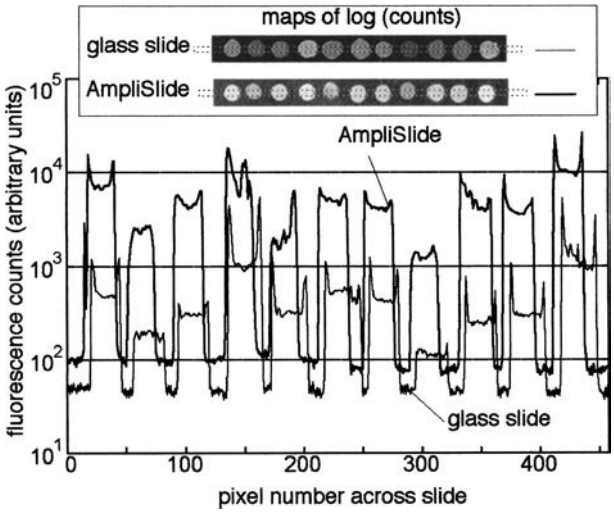

FIG. 3. Illustration of Cy3™ fluorescence enhancement for a DNA hybridization assay on a mirror coated microarray (Amplislide™ thick line), compared to a commercial glass slide (dotted line). The inset gives two grey-level (in logarithmic scale) fluorescence maps of the spots and of the band-shaped areas used to produce the curves. A Scanarray® reader was used to obtain these data.

Figure 1(b) represents the reflection phase φ_{exc} for a four period TiO_2/SiO_2 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 $\theta_{exc}=\theta_{fluo}=0°$ 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 for 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 TiO_2/SiO_2 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

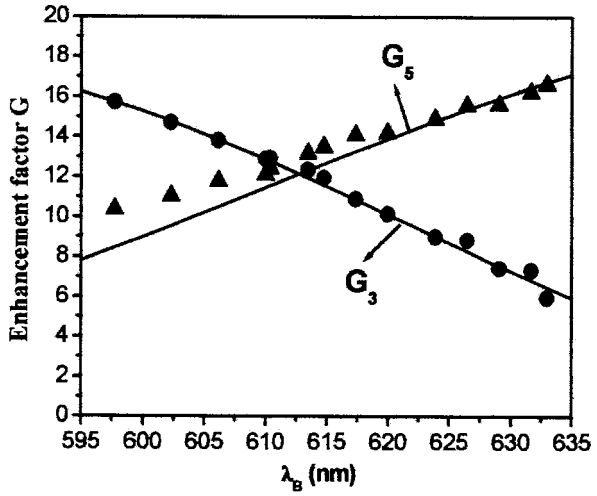

FIG. 4. Theoretical value (solid curves for G_3 and G_5) and experimental measurements of G using BG (triangles) and eosin dyes (circles) for a set of slides with variable Bragg wavelength. Experimental values were normalized versus a glass slide, G theoretical values were divided by $G_{\text{glass}}=0.55$.

assays; details will be reported elsewhere. A representative sample of each microarray is illustrated in Fig. 3: The Cy3 fluorescence counts across the spots shown in the inset tend indeed to be in a ratio $G>10$. A similar trend is obtained for Cy5. These enhancements (up to 15) translate into lower detection threshold and result in identification of genes with low expression levels. They also open other possibilities, such as using less material (probes and targets), using polymerase chain reaction techniques with less amplifications steps, or using less sensitive and more rapid scanners in the field.

It turns out that, with respect to the scope of safely characterizing the Cy3™ and Cy5™ enhancements of mirror slides, analysis based on actual hybridization assays are complex, costly, and not very accurate. We devised a simpler method directly giving fluorescence amplification for both cyanines G_3 and G_5, as follows: We spin coat onto glass or reflective slides a dye-doped layer that thins down to a few nm (so that enhancement curves are not perturbed), selecting a dye with emission spectrum matching that of cyanine 3. Slides were washed with ethanol before another dye-doped coating, matching the Cy5 spectra. The comparison directly measures the gains G of interest, and allows absolute measurements provided both slides are equally coated (same dye concentration per cm^2). In our tests, a thin sol-gel layer[11] annealed at 120 ° C proved an adequate host. Millimolar concentrations of brilliant green (BG) and eosin led to the required FE spectra, with a slightly large linewidth [Fig. 1(c)], leading only to a minute underestimate of the actual performance G if the further spectral filtering action of the scanner is taken into account.

With this efficient method, we further investigated the impact of small fabrication variation between different reflective slides. These variations basically affect the two low and high index layer thicknesses, resulting in variable stopband position and stopband width. However, the width 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 ΔG 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 λ_B was monitored to 1–2 nm accuracy through the position λ_{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 evolution of G_3 (solid curve) and G_5 (dotted curve) as a function of Bragg mirror wavelength (λ_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.

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