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ROBUST NANO CLUSTER LAYERS FOR STRUCTURAL AMPLIFIED FLUORESCENCE BIOCHIPS

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Abstract. Structural enhanced fluorescence (SEF) amplifies fluorescent signals 200 fold in comparison to standard glass slides. The signal-boosting phenomenon is ideally suited for sensitive biochip applications. DNA biochips based on hybridisation analysis need to cope with chemical and thermal stress, therefore a stable multilayer set up and chemically active surface layers were invented. A palladium layer introduces chemical stability and the necessary structural compatibility for the SEF technique. A silver cluster layer enables the excitation of surface plasmons; sandwich layers of metallic glasses establish the proper distance to the fluorophores. The chips are compatible with standard assay procedures of DNA-biochips, and can be reused after hybridisation by stripping in boiling water.

1. INTRODUCTION

A distinct property of metal nano clusters is the strong local electromagnetic field surrounding every cluster due to its resonating electron gas, which alters properties of fluorophores in its vicinity. At distances less than 5 nm metallic quenching dominates, but at greater distances up to 200 optical nm an enhancement of the fluorescence ability of each fluorophore appears. Reason for this effect is the interaction between clusters and fluorophores, which can be described as an analogue to a resonant circuit [1]. During excitation the irradiative light drives the metal clusters plasmons and the fluorophores to act as oscillators. During emission the fluorophores acting as the origins of radiation behave as oscillators with a resonant structure in their vicinity. Surface as well as Mie plasmons are driven by the irradiation, increasing the energy of the local electromagnetic field strongly. Due to a resonant transparent interlayer the fluorophores are at a proper distance (to the cluster layer). Thus the fluorophores are exposed to the enhanced field of the interlayer, resulting in increased absorption probability. Via induction of an additional charge to the fluorophore the cluster layer shifts the relaxation probability towards radiative pathways and speeds up the emission process. It is not yet discovered whether this phenomenon of enhanced fluorescence emission is either uniform or highly directed in space. Cluster layers are eminently polymorph structures. It is vital to obtain a reproducible nano-granular structure and to define the nano-structure versus fluorescence boosting. Responsible for the superiority of the cluster film – fluorophore interaction to other resonant structures is the additional presence of Mie plasmons, into which much more energy can be coupled compared to surface plasmons. These effects enable to manufacture fluorescence-enhanced slides with a theoretical enhancement up to 1000 times [2]. It has to be mentioned that the theoretical considerations are valid for fluorophores with a quantum yield near 1; weaker fluorophores may show

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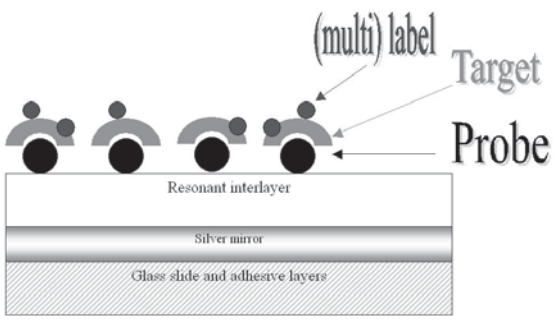

Fig. 1. Basic scheme of a capture type assay on the fluorescent signal enhanced biochips.

much stronger enhancement factors when their quantum yield is shifted towards 1 by the process.

Numerous types of slide based micro arrays are used in clinical genomics, genotyping, identification of proteins and microorganisms. Most of these assays are fluorescence based [3,4]. With a proper set up the SEF technique does not need new designs of protein or DNA assays. Therefore the SEF slides are based on standard microscopic glass slides making them compatible with most arrayers and scanners. The experiments are performed similar to commercial protein or DNA slides, the set up of the slides ensures (with a working biorecognition assay) the fluorescent label to bind at the correct distance to the cluster film, meaning within the oscillating field of the resonant structure. The basic scheme (Fig. 1) illustrates the setup of chips working with this nano-resonant effect. The enhancement of the fluorescent signal due to SEF allows performing assays, which are normally beyond the sensitivity of commercial slides.

The nano cluster layer and thin films of metals needed for the SEF set up are manufactured by sputter coating on standard microscopic glass slides using argon plasma; reactive sputter coating via nitrogen or oxygen plasma produces metallic glasses for the transparent resonant interlayer [5]. The use of sputter coating enables to tune the multilayer system for optimal performance, which is essential for the interlayers optical thickness (optical thickness = thickness of the material times its refraction index). As pointed out above, the enhancement does occur only within narrow zones of distance between the cluster film and the fluorophores, additionally this distance is dependent on the fluorophores excitation and emission characteristics [6]. Therefore, a well-designed chemical bonding of the cluster layer to the chip as well as to the top layer interfacing to the biological fluids is required. A functional architecture for efficient probe immobilization on biochips needs to protect the labile nano-granules from lift-off and chemical degradation.

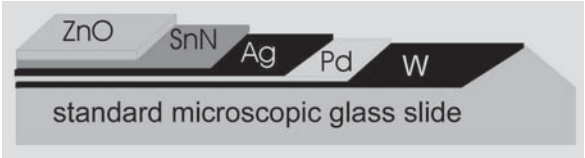

Fig. 2. Set up of the multi layered SEF slides.

2. MATERIALS AND METHODS

2.1.Chip set up.

The deposition of metals and metallic glasses or cluster layers was done using an EMITECH K675X sputter coater on isopropanol cleaned microscopic glass slides. For each step three 2-inch targets of metal are used, as well as a special designed rotating table in the vacuum chamber of the sputter coater providing high homogeneity of each layer. First a tungsten film is applied to serve as adhesion layer (sputter gas: argon at $1 \cdot 10^{-2}$ mbar, 450 mA, 3 minutes) due to the fact that especially silver binds poorly to glass, and also palladium does not attach efficiently enough for the chemical and thermal stress of hybridisation assays. To roughen the surface and to protect the tungsten from assay chemicals palladium is sputtered on top of the tungsten layer. (sputter gas: argon at $1 \cdot 10^{-2}$ mbar, 450 mA, 1 minute). Silver is deposited on top to serve as plasmon source (sputter gas: nitrogen at $1 \cdot 10^{-2}$ mbar, 450 mA, 3 minutes), the 'clustering' is achieved due to the given palladium nano-structure and finalized via curing at temperatures of 200 to 300 °C. The inert distance layer is set up as a combination of the metal glasses tin nitride (target: tin, sputter gas: nitrogen at $2 \cdot 10^{-2}$ mbar, 450 mA, 1 minute) and zinc oxide (target: zinc, sputter gas: oxygen at $2 \cdot 10^{-2}$ mbar, 450 mA, 3 minutes). The distance layer must on one hand supply the correct distance of silver clusters to fluorophores and on the other hand protect the sensitive silver layer from chemical destruction. Fig. 2 shows the set up and sequence of layers of such multi layered SEF slides.

2.2. Surface activation:

It has to be mentioned that several other surface activation procedures like poly-L-lysin or avidin coating as well as EDC activation were investigated beside the described steps [7]. Although in certain steps or for protein slides successful these techniques proved to be incompatible with the needs of the hybridisation steps.

Cystamine "semi-activation". The slides are placed inside a slide holder, and incubated in a 10^{-4} M Cystamine solution (Cystamine in water) over night. After rinsing with water the slides are dried in an air flow or by centrifugation.

Silane activation. The slides are placed inside a slide holder, which is positioned in a vacuum chamber as well as 10% silane (amino or epoxy-silane) in Toluene. Silane and slides are exposed to vacuum, the chamber is sealed off resulting in silane vapors filling the chamber and attaching to the slides surface. Subsequently, the slides are washed with Toluene and iso-propanol to remove excess silane multilayers and heated for 30 minutes at 105 °C to covalently attach the silane molecules to the surface.

Divinylsulfone (DVS) activation. Except the Epoxy activated slides 2.1 and 2.2 result in accessible Amino-groups at the surface requiring an additional activation step: Therefore the slides are incubated in 0.5% DVS in 0.1M Na_2CO_3/$NaHCO_3$ pH 9 buffer for 15 min at room temperature. After rinsing with 0.1M Na_2CO_3/$NaHCO_3$ pH 9 the slides are dried as described above.

2.3. Immobilization

2.3.1. For testing of the enhancement itself several fluorophores including Cy3, Cy5, rhodamine 6G and fluoresceine were adsorbed onto the slides without using further steps except removing of excess fluorophore via rinsing in water and subsequent drying of the slides (as described above).

2.3.2. For testing the efficiency of the surface activation fluorescently (Cy3 or Cy5) labelled BSA or NHS-activated fluorophores dissolved in water in a microtiter plate are dotted by a GMS 417 arrayer onto the slides surface. The slides are incubated at 60 degrees in a humid chamber, washed with water and subsequently blocked with 0.1% milk powder in 0.1·SSC. Excess protein is removed by rinsing with water and finally the slides are dried via air flow or centrifugation.

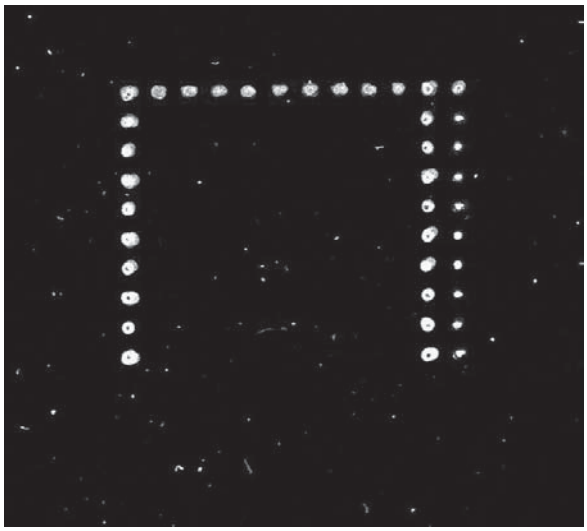

Fig. 3. Scan of a BSA labeling control enabling to find the array, its orientation and in which row hybridization does occur.

2.3.3. For testing the usability for hybridisation DNA-probes containing an aminolinker dissolved in 0.05 M $NaHCO_3$/Na_2CO_3 pH 9.0 buffer in a microtiter plate are dotted, incubated, washed and blocked as described in 2.3.2.

2. 4. Hybridisation test [8]

A Teflon coverslip is placed on the arrays and 30 µl of fluorophore-labelled DNA (1-50 pmol/µl) in hybridisation buffer (3·SSC or commercial buffers) is pipetted next to the coverslip, resulting in sucking the solution under the coverslip. Incubation at hybridisation temperature is done for 1-2 hours, subsequently the slides are washed with washing buffers (0.1-2·SSC, 0-0.2% SDS depending on the hybridisation protocols). Finally the slides are dried via centrifugation.

2.5. Scanning

All scans were done on a commercial fluorescence slide scanner (GSI Lumonics ScanArrayer Lite). Fig. 3 shows such a scan thereby combining the approaches 2.3.2 and 2.3.3. All bright dots are Cy 5 labelled BSA scanned at a laser power and a PMT-voltage setting of 80%. These dots enable to read out the array easily (even if there was no successful hybridisation). The horizontal and vertical pattern of the BSA dots even allows to read out the

array without using any software. With such a set-up also the critical steps of a hybridisation protocol can be identified by tracking if and when the original label disappears.

3. RESULTS AND DISCUSSION

Compared to slides set up without the palladium layer the fluorescence enhancement was significantly higher for thermally uncured slides. For cured slides the enhancement was just slightly improved, but for all approaches the mechanical and chemical stability of the palladium containing slides proofed superior.

The surface activation with Cystamine enabled to bind activated fluorophores covalently. Different to expectations the Cystamine bond was well stable at higher temperatures, leaking could not be detected up to 70 degrees Celsius. However for certain fluorophores, among them Cy5 and Ru(1-10-phenanthroline)3 Cl2, the enhancement of fluorescence decreased significantly. As reason for that behaviour was found that the fluorophores did not homogeneously attach to the surface. DVS activation of the Cystamine surface could improve the homogeneity of the fluorophore attachment, but still the results were poor compared to non-activated surfaces. Another disturbing effect for the cystamine activation was observed when copying standard commercial protocols using their commercial "dotting" or "spotting" solutions. Under high salt conditions comparable to 3-5*SSC no binding at all was observed.

All silane activation attempts of the Zinc oxid or Tin nitride surfaces gave very poor results. From the activated fluorophore attachment it was concluded that the silanisation efficiency on these surfaces is at least 100 times lower compared to glass. A solution to that problem was the introduction of a new top layer. Indium tin oxide (ITO) as well as Tin oxide ((target: tin, sputter gas: oxygen at $2 \cdot 10^{-2}$ mbar, 450 mA, 45 seconds) showed much better silane activation. However the superior stability of the SnN/ZnO layer combination was lost. Additionally sputtering of these materials is very tricky (ITO) or simply more time consuming (ITO and SnO), due to the fact that targets easily overheat and melt, which can only be avoided by sputtering several cycles with intermediate cooling times. A more promising approach, which is under investigation at the moment, will be to set up a 3-component layer of SnN/ZnO/SnO whereby the ZnO layer thickness is reduced to approx. 70% of its original height, and the deposition of SnO results in the original optical height of the slides. This set up should include the superior stability of the ZnO containing slides as well as the superior silanisation efficiency of SnO.

Good results of the DVS activation were soon plagued by irreproducible results. It was shown that the binding activity of the surface decayed in time, which explained quite different results of the same experiments. The solution to this problem was to activate the slides freshly before the dotting procedure, keeping the "storage time" of the so activated surface well below 1 hour. Sticking to that procedure the DVS activation gave excellent results.

4. CONCLUSION

A quick and reproducible production protocol for robust surface enhanced fluorescence chips for DNA hybridization assays was established. Several possibilities of activating the surface for covalent immobilization of probes have been successfully tested. A new generation of surface composition and activation should additionally enable to store the slides between activation and use for considerable times, making them more useful for commercial applications.

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