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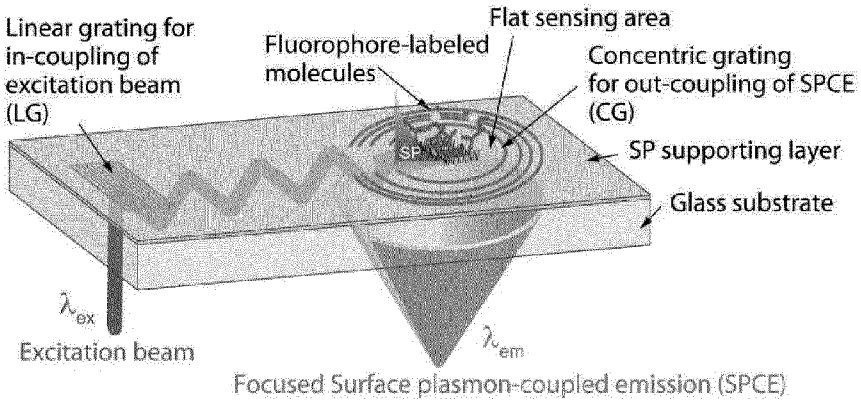
(54) Title: COMPACT PLASMON-ENHANCED FLUORESCENCE BIOSENSOR

Fig. 2a



(57) Abstract: The present invention related to a compact biosensor chip for fluorescence assays enabling detection of trace amounts of biomolecules. It employs surface plasmons for the enhanced excitation and efficient collecting of fluorescence light in order to amplify detected fluorescence light intensity. The integration of key optical elements onto the sensor chip by using concentric and linear gratings allows simplifying of design of overall biosensor setup.

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COMPACT PLASMON-ENHANCED FLUORESCENCE BIOSENSOR

FIELD OF THE INVENTION

The present invention is related to the signal amplification and enhancing the sensitivity in fluorescence-based assays by using surface plasmon optics combined with diffractive optical elements. It enables design of a compact biosensor for the detection of trace amounts of biomolecules in liquid samples.

BACKGROUND OF THE INVENTION

Sensitive, portable and cost-effective biosensors are intensively pursued for the detection of chemical and biological analytes in important areas of point-of-care medical diagnostics, food control, security and environmental monitoring. Fluorescence-based detection is one of the mostly used methods due to its intrinsic capability for sensitive detection. In order to further advance its performance, various approaches were researched including surface plasmon-enhanced fluorescence (PEF) (J. R. Lakowicz, Plasmonics, 1, (2006) 5-33; Stewart, et al., Chemical Reviews, 108, (2008) 494-521). Surface plasmons (SPs) are optical waves that originate from coupled collective oscillations of electron density at metallic surfaces. These waves exhibit strongly increased intensity and highly confined profile of electromagnetic field. The coupling of fluorophore labels with surface plasmon field offers an attractive means for the amplification of fluorescence signal-to-noise-ratio in bioassays through the combination of increased fluorophore excitation rate, decreased background signal, directional fluorescence emission and enhanced fluorophore quantum yield. Up to now, two main approaches that rely on SPs propagating along continuous metallic films were pursued and implementated to PEF biosensors. In surface plasmon-enhanced fluorescence spectroscopy (SPFS) (Dostalek and Knoll, Biointerphases, 3, (2008) 12-22; Liebermann and Knoll, Colloids and Surfaces a-Physicochemical and Engineering Aspects, 171, (2000) 115-130; Herrmann, et al., US 6194223 B1), the binding of fluorophore-labeled molecules to biomolecular recognition elements attached on a metallic sensor surface is probed by the enhanced field intensity of SPs at the wavelength matching the absorption band of used fluorophore labels. This method takes advantage of the increased excitation rate of fluorophores that is directly translated to an enhanced fluorescence signal, see Fig. 1. In surface plasmon-coupled fluorescence emission (SPCE) (J. R. Lakowicz, et al., Biochemical and Biophysical

Research Communications, 307, (2003) 435-439), highly directional fluorescence light beam emitted via SPs at the emission wavelength is detected. Bulk optical prism elements (Yuk, et al., Biosensors & Bioelectronics, 25, (2010) 1344-1349; J.R. Lakowicz, et al., US 20050053974; Gryczynski, et al., US 20090218516 A1), and metallic diffraction gratings (Kitson, et al., Optics Communications, 122, (1996) 147-154) were reported for the extracting the fluorescence light trapped by SPs to the radiation propagating to specific directions in the far field. A sensor chip with series of hemispherical dielectric elements was proposed for disposable optical biosensor chips with SPCE (Yuk, et al., Biosensors & Bioelectronics, 25, (2010) 1344-1349). Owing to the confined profile of SP field at the metallic surface, only the fluorescence signal originating from fluorophores in close proximity to the metallic surface (distance up to ~100 nm) is detected by SPFS and SPCE methods, leading to a greatly suppressed background.

Various dielectric diffraction elements such as concentric gratings were used for manipulation of light. However, they were not combined with metallic structures for the amplificiation of fluorescence signal by using surface plasmons. These include the following works:

US2003043475A1 describes a compact diffraction type lens which can converge two wavelengths of light onto their corresponding optical recording media having disc thickness values different from each other.

US2010091370A1 describes an optical resonator structure including a substrate; a center disc formed on the substrate; a plurality of concentric grating rings surrounding the center disc, the concentric rings spaced apart from the center disc and from one another by regions of lower index of refraction material with respect thereto

US2012038918A1 describes an optical element which has the dual functionalities of a grating and a Fresnel lens. The grating side may be coated with a dielectric film.

EP1644871B1 discloses an optical security element having a substrate layer in which a relief structure defined by relief parameters is shaped out in a surface region.

US20060018021A1 discloses an optical security element including an optical grating structure which exhibits pleochroic properties when rotated or viewed from changing observation locations. The optical grating structure is formed from a plurality of selectively arranged grating elements.

SHORT DESCRIPTION OF THE INVENTION

The invention describes a method and apparatus for an amplification of fluorescence signal in assays for the detection of biomolecules through simultaneous excitation and collection of emitted fluorescence light by using surface plasmons. It is based on a planar sensor chip (biochip) with diffractive optical elements (DOE) combined with Kretschmann configuration for the excitation and back-coupling of surface plasmons into the substrate. Diffractive optical elements are prepared on the biochip surface and include a concentric relief grating (CG) and linear relief grating (LG). CG is designed to function as a holographic lens for the imaging of highly directional surface plasmon-coupled fluorescence emission from fluorophore labels to a spot below the biochip where a detector is placed. Additional LG element is employed for the generating of surface plasmons at the excitation wavelength in order to increase the excitation rate of fluorophores attached to biomolecules that are specifically captured on the biochip. The reported approach offers the advantage of increased intensity of fluorescence signal and reduced background. The implementation of key optical elements on the biochip surface in form of DOEs simplifies design of the overall biosensor optical setup which does not require optical matching of a sensor chip to other bulk optical elements (such as prism or microscope lens), and the biochip can be fabricated by mass production-compatible technologies such as nanoimprint lithography.

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns a method and implementation of combined surface plasmon-enhanced fluorescence spectroscopy (SPFS) and surface plasmon-coupled fluorescence emission (SPCE) for sensitive detection of molecular and biological analytes. It allows simplification of the biosensor design that is suitable for portable compact devices. It relies on diffractive optical elements integrated to the biochip for the coupling of an excitation beam to surface plasmons (linear grating - LG) and for the imaging of surface plasmon-coupled fluorescence emission light to a spot below the biochip where a detector is placed (concentric grating - CG), see Fig.2. These elements replace traditionally used bulk optical components such as prisms and microscope lenses and avoid the necessity of their optical matching to the sensor chip. The whole biochip can be fabricated by mass production-compatible technologies such as nanoimprint lithography, hot embossing or injection molding to inexpensive polymer

materials. In addition, it enables multi-analyte detection of molecular analytes by using microarrays since CG acts as a diffractive lens. Owing to this feature, the fluorescence light intensity emitted from different areas on the biochip can be projected to spatially separated spots in the image plane and independently detected with a spatially sensitive detector (e.g., charge-coupled device – CCD).

There is thus provided according to one aspect of the invention a flat dielectric substrate carrying a relief concentric grating element.

The dielectric substrate may be constructed of a material that is transparent to light wavelengths that are suitable for the excitation and emission of fluorescence light in visible and near infrared part of spectrum, such as polycarbonate, quartz or glass.

According to another aspect of the invention there is provided a flat substrate, comprising at least one linear grating element and at least one concentric grating element.

According to yet another aspect of the invention there is provided a structured polymer layer on the substrate, wherein said relief concentric grating has a depth of at least 10 nm and period between 0.2 and 1 micrometer.

According to yet another aspect of the invention there is provided a polymer layer, wherein said polymer layer is structured by using interference lithography, electron beam lithography or nanoimprint lithography.

According to yet another aspect of the invention there is provided a sensor chip comprising a transparent glass or plastic substrate and on the outer surface a polymer layer with at least one concentric grating element and at least one sensing area is prepared.

According to yet another aspect of the invention there is provided a chip, further comprising a metal layer on the sensing area in the middle of the concentric grating.

According to yet another aspect of the invention there is provided a chip, wherein said metal is gold, silver, or aluminum.

The metal is to be selected to support surface plasmons in the visible and near infrared part of spectrum.

According to yet another aspect of the invention there is provided a chip, wherein said metal layer on a dielectric substrate exhibits a thickness between 30 and 70 nm and support surface plasmons.

The thickness of the metal layer is in such a manner to support surface plasmons at the outer metal surface that are leaky into the dielectric substrate.

According to yet another aspect of the invention there is provided a chip, wherein biomolecular recognition elements for selected chemical or biological compounds are attached to the metallic surface on the sensing area.

The biomolecular recognition element is able to capture target analyte. Various types of biomolecular recognition elements are known in the art, e.g. antibodies, aptamers, peptides, molecularly imprinted polymers, etc. To enable analyte detection, a biomolecular element specific to the target analyte is immobilized in the sensing area on the biochip. It needs to be assured that the biological activity of the immobilized biomolecular recognition elements is conserved and the biochip surface exhibits non-fouling properties. The possibility to regenerate the biomolecular recognition elements (i.e., break their complex with the analyte molecules and make them available for another use) can be considered.

In general, methods for the immobilization of biomolecular recognition elements on metal films exploit physicochemical interactions such as chemisorptions, covalent binding, electrostatic coupling, and high-affinity molecular linkers in multilayer systems (e.g., streptavidin–biotin, proteins A or G, and complementary oligonucleotides) and photo-immobilization (e.g., albumin conjugated with aryldiaziridines as a photo-linker). For example, n-alkylthiols or disulfides may spontaneously self-assemble on gold into well-ordered arrays.

According to yet another aspect of the invention there is provided a chip, wherein biomolecular recognition elements are covalently attached by using self-assembled monolayer (SAM), in polymer brush or hydrogel film.

SAMs have been employed in many immobilization methods for spatially controlled attachment of biomolecular recognition elements to surfaces of sensors. To provide a desired surface concentration of biomolecular recognition elements on metal, mixed SAMs of long-chained (nD12 and higher) n-alkylthiols terminated with functional group for further attachment of biomolecular recognition elements and short-chained alkylthiols for a non-fouling background have been developed. To deliver molecular recognition elements to different sensing areas, the immobilization chemistry needs to be spatially controlled. Most of the current technologies of proteins arrays are based on the surfaces and formats that were earlier developed for DNA arrays. Most DNA array production techniques were developed for glass supports, but they can be tailored to noble metal surfaces with appropriate immobilization chemistries. Combination of SAMs with covalent coupling of biomolecular recognition elements or

non-covalent streptavidin–biotin system as a linker for attachment of biotinylated biomolecular recognition elements are most frequently used approaches to development of protein arrays on metal.

According to yet another aspect of the invention there is provided a chip, wherein fluorophores are employed as labels and after their capture at the sensing area they emit light via surface plasmons that are out-coupled to the substrate by reverse Kretschmann configuration. The characteristic surface plasmon-coupled fluorescence emission cone is totally internally reflected at the bottom substrate interface and hits the concentric grating element at the top biochip surface.

According to yet another aspect of the invention there is provided a chip, wherein the concentric grating focuses the light beam emitted from the sensing area at a narrow spot below the biochip.

According to yet another aspect of the invention there is provided a chip, wherein the concentric grating focuses the light beam emitted from different locations on the sensing area to different spots below the biochip.

According to yet another aspect of the invention there is provided a chip, wherein the concentric grating exhibits a relief modulation with the depth between 20 and 200 nm and the relief modulation with period between 200 nm and 1 μm is chirped and serves as a diffractive lens.

According to yet another aspect of the invention there is provided a chip, wherein the concentric grating that is coated by a metal layer to increase its diffraction efficiency.

According to yet another aspect of the invention there is provided a chip, wherein the concentric grating exhibits sinusoidal or blazed relief modulation providing high diffraction efficiency.

According to yet another aspect of the invention there is provided a chip, wherein the fluorophore labels at the sensing area are organic chromophores or quantum dots emitting fluorescence light in visible or near infrared part of spectrum.

According to yet another aspect of the invention there is provided a chip, wherein said fluorophores are exited with a light beam at lower wavelength than their emission wavelength.

According to yet another aspect of the invention there is provided a chip, wherein excitation light is monochromatic and it is coupled to the biochip by a linear grating, propagates in the biochip substrate and hits the sensing area.

According to yet another aspect of the invention there is provided a chip, wherein the said excitation beam hits the sensing area at the angle at which surface plasmons are resonantly excited providing enhanced field intensity at the top of the sensing area.

According to yet another aspect of the invention there is provided a chip, wherein the excitation beam excites the fluorophore labels adhered to close proximity to the sensing area.

According to yet another aspect of the invention there is provided a chip, wherein the linear grating exhibits a periodic relief corrugation with the modulation depth between 20 and 200 nm and period between 200 nm and 1 μm.

According to yet another aspect of the invention there is provided a chip, wherein the periodic modulation of linear grating is sinusoidal or blazed and provides high diffraction efficiency.

According to yet another aspect of the invention there is provided a chip, wherein the linear grating is coated by additional metal layer to enhance its diffraction efficiency.

According to yet another aspect of the invention there is provided an apparatus for detecting fluorescence in biochemical assays by combined surface plasmon-enhanced fluorescence and surface plasmon-coupled emission methods, comprising:

a) a chip as described above with at least one linear, at least one concentric gratings and layer architecture supporting surface plasmons; and

b) an excitation source that emits a light beam incident at the linear grating and that is capble exciting fluorophores in the sensing area by surface plasmons; and

c) a light detector arranged to selectively detect fluorescence light that is generated by excited fluorophores, emitted in form of surface plasmon-coupled emission, and imaged to a spot below the chip.

According to yet another aspect of the invention there is provided an apparatus, comprising the chip as descripted above as an insertable element.

According to yet another aspect of the invention there is provided an apparatus, wherein the chip is a disposable element

According to yet another aspect of the invention there is provided a method for measuring the concentration of an analyte in a sample by combined surface plasmon-enhanced fluorescence and surface plasmon-coupled fluorescence emission comprising the steps of:

a) contacting a sensor chip as described above with a sample enabling the capture of target analyte on a surface and decorating the captured target analyte with fluorophore labels by using sandwich, displacement or competitive assays;

b) exposing the fluorescence label to excitation electromagnetic energy in an amount sufficient to achieve surface plasmon-enhanced excitation; and

c) measuring the emitted fluorescence light intensity in form of surface plasmon-coupled emission that is out-coupled from the biochip and imaged to a detector by using the concentric grating

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the geometry supporting surface plasmon modes on a continuous metallic film that interact with a fluorophore (left) and a Jablonski diagram with plasmon-mediated transitions in the fluorophore (right).

Figure 2a shows a schematic of the biochip carrying diffractive optical elements (DOE) for the in-coupling of the excitation laser beam to the biochip and subsequent excitation of surface plasmons (LG) and for the imaging of surface plasmon-coupled fluorescence light to a detector (CG). Figure 2b depicts the side view of the biochip and shows the propagation of the excitation and fluorescence beams.

Figure 3 depicts schematics of the preparation of the concentric grating element (CG) by using sequential recording into a photoresist based on interference lithography

Figure 4 shows the dependence of the concentric grating element (CG) period on the distance from its center obtained from simulations (line) and measured on prepared biochips (squares).

Figure 5 depicts a schematic of UV imprint lithography employed for the transfer of LG and CG elements to the biochip.

Figure 6 depicts an optical setup for the observation of imaging properties of concentric (CG) element.

Figure 7 depicts an optical setup employed for a model immunoassay experiment based on the biochip with plasmon-enhanced fluorescence detection.

Figure 8 shows the dependence of emission probability from a randomly oriented dipole via surface lossy waves (quenching), surface plasmons that are out-coupled into the substrate (SPCE) and optical waves propagating in free space. Gold layer with the thickness of 50 nm sandwiched between a substrate (n_p) and water (n_s) with a dipole emitting at the wavelength λ_{em}=670 nm are assumed.

Figure 9 shows simulated angular dependence of the fluorescence intensity F of SPCE that is back-coupled to the substrate from a randomly oriented dipole at the distance d=20 nm from the gold surface at the wavelength λ_{em}=670 nm (solid line), and the electric field intensity enhancement $|E/E_0|^2$ at d=20 nm provided by the excitation of SPs at the wavelength λ_{ex}=633 nm (dashed line).

Figure 10 shows simulations of -1st order diffraction efficiency of a sinusoidal concentric grating (CG) element (at the fixed location with Λ=346 nm) depending on the grating depth for the TM polarized light beam incident at the emission angle θ_{em}=71 deg and emission wavelength of λ_{em}=670 nm.

Figure 11 shows simulations of +1st order diffraction efficiency of a sinusoidal linear grating (LG) element for the normal incident TM polarized beam at the wavelength of λ_{ex}=633 nm and the period of Λ=437 nm dependent on the grating depth.

Figure 12a) shows top view of the prepared biochip carrying LG and CG diffractive elements (left) and measured spatial distribution of SPCE cone that is out-coupled from the biochip by using a CG element at increasing distance below the biochip D=1, 5, 10 and 15 mm (right). b) The cross-section of the fluorescence spot at the distance of D=15 mm from the bottom of the sensor for the angular width of individual CG segments are δ=3 and 10 deg.

Figure 13 shows measured kinetics of a-mIgG binding to the surface with immobilized mIgG (specific affinity partner) and rIgG (reference molecule) on the prepared biochip with CG and LG elements. The inserted graph shows the magnified fluorescence intensity $F(t)$ for the concentration from 30 pM to 1 nM

Figure 14 shows calibration curve of the developed biochip for the detection of a-mIgG analyte fitted with a linear function. The baseline noise and LOD are indicated.

EXAMPLES

1. Materials and methods

1.1. Materials

Polydimethylsiloxane (PDMS) prepolymer and its curing agent were purchased from Dow Corning (SYLGARD® 184). Poly(methyl methacrylate) (PMMA) was from Sigma-Aldrich Handels (Austria). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbo cyanine, 4-chlorobenzenesulfonate salt (DiD) was from Invitrogen. This dye exhibits

the absorption and emission bands at wavelengths of λ_{ab}=644 nm and λ_{em}=665 nm, respectively, and it was dispersed at the concentration of 700 nM in a toluene with PMMA (1.4 wt.%). Dithiolalkanearomatic PEG 6-COOH (COOH-thiol) and dithiolalkanearomatic PEG3-OH (PEG-thiol) are from SensoPath Technologies (USA). Phosphate buffered saline (PBS) with pH 7.4 was obtained from Calbiochem (Germany). PBS-Tween (PBS-T) buffer was prepared by adding 0.05% of Tween20 (Sigma-Aldrich, USA) to PBS buffer solution. Anti-mouse mIgG (a-mIgG) and mouse mIgG (mIgG) were from Molecular Probes (USA). a-mIgG molecules were labeled with Alexa Fluor 647 with the dye-to-protein molar ratio of 4.5. This dye exhibits the absorption and emission wavelengths of λ_{ab}=650 nm and λ_{em}=668 nm, respectively. Rabbit IgG (rIgG) was from Abcam (USA). 10 mM acetate buffer (ACT) with pH 5.5 was prepared in house. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were from Pierce (USA). Ethanolamine (Sigma-Aldrich, USA) was dissolved in water at 1 M concentration with the pH of the solution adjusted to 8.5 with sodium hydroxide.

1.2. Biochip preparation

Interference lithography (holography) was used for the preparation of master gratings. Two different masters with linear sinusoidal relief grating (LG) and concentric relief chirped grating (CG) were prepared and characterized by atomic force microscope. LG exhibited the period of Λ=436 nm and depth of 100 nm. CG was composed of 120 or 36 segments carrying chirped gratings with the varied period between Λ=365 and 313 nm at the distance from the center of CG of 4.7 and 8.5 mm, respectively, and with the average depth of 110 nm. Fig. 3 shows the preparation of CG and LG elements by using interference lithography. Triangular sections on a photoresist-coated substrate was sequentially exposed to an interference field with an angular step δ (δ=10 or 3 deg) in order to approximate the concentric grating by a set of chirped linear gratings. The interference pattern formed at the intersection of a collimated and converging laser-beams was tuned by a cylindrical lens in order to match the desired dependence of the period that was determined from simulations (see Fig. 4). After the exposure of the photoresist to the interference field, the gratings were subsequently etched with a developer AZ-303, rinsed with water and dried. In order to prepare multiple replicas, the master grating was casted to PDMS and

transferred into a UV curable polymer followed by the gold deposition as shown in Fig. 5. Gold layers with the thickness of 50 and 200 nm were deposited on the flat sensing area and on the surface of LG and CG elements, respectively, by sputtering (UNIVEX 450C, Leybold Systems, Germany).

1.3. Simulations

Finite element method (FEM) grating solver DiPoG (Weierstrass Institute, Germany) was used for the calculation of the diffraction efficiency. The chirped grating and the overall biochip geometry was designed by using a ray-tracing tool Zemax (Radiant Zemax, USA). For the simulation of surface plasmon-coupled emission, home-developed scripts based on the CPS-model were used as described in our previous works.

1.4. Surface modification

For the observation of imaging properties of CG element, 20 nm thick PMMA layer doped with DiD dye was spin-coated on a gold-coated surface of a sensing area and dried overnight at the room temperature. In order to function as a biosensor, the sensing area with 50 nm thick gold layer was modified with a mixed thiol self-assembled monolayer (SAM), and IgG molecules were covalently attached to SAM carboxylic functional moieties. The biochip was firstly immersed in a mixture of PEG-thiol and COOH-thiol dissolved in ethanol (molar ratio of 9:1 and total concentration of 1 mM) overnight at room temperature. Afterwards the biochip was rinsed with ethanol and dried in a stream of nitrogen. mIgG antibodies were immobilized by amine coupling. Carboxylic terminal groups of COOH-thiol were activated by EDC and NHS solution (concentrations in deionized water of 75 and 21 mg/mL, respectively) for 15 min, followed by the incubation with mIgG dissolved in ACT buffer at the concentration of 50 µg/mL for 90 min. Unreacted active ester groups of the COOH-thiol were passivated by 20 min incubation in ethanol amine solution. In a control experiment, rIgG with the same concentration was immobilized instead of mIgG.

1.5. Optical setup for testing of biochip performance

Diffracted and out-coupled SPCE beam propagating below the biochip was made scattered at the rough surface of a diffuser which was placed at a distance *D* from the chip. The spatial distribution of scattered fluorescence light was imaged to an

electron multiplying charge-coupled device (EM-CCD iXon+885, Andor Technology, Ireland) by a camera lens (UNIFOC 58, Schneider Kreuznach, Germany) as seen in Fig. 6. A set of filters including notch filter (XNF-632.8-25.0M, CVI Melles Griot, Germany) and band-pass filter band-pass filter (670FS10-25, LOT-Oriel, Germany) was used in order to suppress the background. For the investigation of surface plasmon-enhanced intensity of the out-coupled SPCE light beam, the excitation light beam was made incident from the bottom of the biochip at LG. The diffracted light beam propagated in the biochip and excited SPs at the sensing area with 50 mn thick gold film. As a reference, the fluorescence was excited with an excitation light beam directly incident at the biochip surface from the top through water.

In a model bioassay experiment, the SPCE signal that was focused by the CG element was collected by a microscope objective (NA=0.85, NT38-340, Edmund Optics, Germany) and its intensity F was detected by a photomultiplier tube (H6240-01, Hamamatsu, Japan) which was connected to a counter (53131A, Agilent, USA) (Fig.7). The identical set of filters as in the measurement of spatial distribution of fluorescence light was used to surpress the background signal.

2. RESULTS AND DISCUSSION

2.1 Surface plasmon-mediated fluorescence excitation and emission

As seen in simulations based on Chance-Prock-Silbey model as shown in Fig. 8, the coupling of a fluorophore with surface plasmons strongly depends on the distance from a metal surface d. For a gold surface in contact with an aqueous medium (n_s=1.33) and a fluorophore represented as a randomly oriented dipole, the simulations reveal that the majority of emitted light intensity at the wavelength around λ_{em}=670 nm is collected by propagating SPs at the distance around d~20 nm. Below this distance, the emission is strongly quenched by Förster energy transfer while at higher distances d>50 nm the majority of light intensity is emitted to waves propagating in free space.

For a thin gold film attached to a dielectric substrate, the fluorescence light emitted via SPs is transmitted through the metal film and forms a characteristic SPCE cone which is directional in polar angle θ_{em} and isotropic in azimuth angle ϕ, see Fig. 8. In Fig. 9, we simulated the dependence of the fluorescence intensity F on polar angle θ assuming a randomly oriented dipole to be located on the top of 20 nm thick spacer layer (d=20 nm) on a 50 nm thick gold layer. The SPCE intensity peaks at the polar

angle θ_{em}=72 deg that is the plasmon resonance angle at the wavelength λ_{em}=670 nm. For the identical layer structure, electric field intensity enhancement $|E/E_0|^2$ at d=20 nm was calculated upon the excitation of SPs by a light beam hitting the gold surface from the substrate under the angle of incidence θ_{ex} at the excitation wavelength λ_{ex}=633 nm. The results in Fig. 9 reveals that strong field intensity builds up at the angle θ_{ex}=74 deg where the resonant condition for the excitation of SP at λ_{ex} is fulfilled.

2.2 Biochip design and development

As seen in the Fig. 2, the biochip composed of a BK7 glass slide with a sensing area coated by a 50 nm thick gold film and attached biomolecular recognition elements for the specific capture of fluorophore-labeled target molecules on its top. In order to collect the fluorescence light intensity emitted in form of SPCE cone at the fluorophore emission wavelength λ_{em}=670 nm from the sensing area, a relief concentric diffraction grating (CG) around the sensing area was used. The grating was coated with 200 nm thick gold film acts as a diffraction lens which images the SPCE cone emitted from the sensing area to a spot below the biochip. The SPCE cone propagating at polar angles between θ_{em}=67-77 deg (see Fig. 9) in the glass substrate is totally internally reflected at the bottom glass surface, hits the surface of CG element and is diffracted to a converging wave that focuses at a desired distance D below the biochip where a detector is placed.

The dependence of the grating period Λ on the distance from the grating center r was determined by simulations (shown in Fig. 4) in order to diffract the incident SPCE beam to the -1st order that propagates away from the surface through the glass substrate and converges at D=10 mm. Sinusoidal relief corrugation was used with the average modulation depth of 120 nm which, according to finite element method (FEM) simulations, provides the maximum diffraction efficiency of 81 % (see Fig. 10). In order to couple the excitation beam at the wavelength matching the absorption band of fluorophore, additional linear grating (LG) was employed. LG with the period of Λ=437 nm was used which allowed the coupling of normal incident monochromatic beam to a wave that propagates along in the glass substrate and totally internally reflects at bottom and top interfaces with the angle θ_{ex}=74 deg. When hitting the sensing area with 50 nm thick gold film and aqueous sample on the top, the light beam generates SPs at its surface as this angle coincides with SP resonance angle. LG exhibited

sinusoidal profile with the modulation depth around 110 nm. For 1st diffraction, simulations predict moderate maximum diffraction efficiency of 34 % (Fig. 11).

2.3 Imaging properties of CG and LG elements

The imaging properties of CG were observed by the measurement of spatial distribution of fluorescence light intensity out-coupled from the biochip at different distances *D* below the biochip as seen in Fig. 6. The sensing area of the chip carrying CG (see Fig. 12a) was coated with a PMMA layer loaded with a DiD dye (exhibiting similar characteristics as Alexa Fluor 647 used in further biosensor experiments) with the thickness 20 nm. In the used setup, the sensing area was brought in contact with water and exposed to a normal incident laser beam at the wavelength λ_{ex} illuminating the area of around ~1 mm^2. As seen in Fig. 12a, the intensity of fluorescence light exhibits characteristic ring distribution with a decreasing diameter when increasing the distance *D*. The fluorescence beam is focused and reaches a minimum diameter at the distance of *D*~15 mm which is close to that predicted by simulations. The diameter of the spot at which the fluorescence light is confined depends on the finess with which CG is prepared. As Fig. 12b shows, full width at half maximum (FWHM) of the focused beam crossection of 0.7 mm was observed for the angular width of individual CG segment of δ=3 deg which is smaller than 2.3 mm measured for the wider segments with δ=10 deg. Let us note that additional broadening of the focused fluorescence beam area is caused by finite area on the sensing area that is illuminated by the excitation beam and by chromatic abberation of the CG lens. The fluorescence intensity detected in the focal plane at the wavelength λ_{em}=670 nm is increased when dyes dispersed on the surface are excited with the enhanced intensity of surface plasmons at the excitation wavelength λ_{ex}=633 nm. In further experiments, the excitation of the fluorescence light via surface plasmons at the wavelength λ_{ex}=633 nm was used. In this configuration, the laser beam was coupled to the biochip by the LG, propagated along in the chip substrate and excited SPs on the sensing area at the resonance angle θ_{ex}. Only moderate enhancement of the fluorescence light collected by SPCE ~2.3 was observed with respect to that measured for normal incident excitation beam from the top (data not shown). This is due to the relatively low diffraction efficiency of the LG element (at least a factor of 0.34) and attenuation of the excitation beam by multiple reflections at the surface between BK7 glass substrate and 200 nm thick gold (at least a fact of 0.6) which reduced the excitation light intensity by

a factor >5. This values is in agreement with the the electric field intensity enhancement $|E/E_0|^2$=17 predicted by the simulations in Fig. 9.

2.4 Demonstration of model bioassay with SPFS/SPCE readout

In order to demonstrate the potential of developed biochip with combined SPFS and SPCE for sensitive detection of biomolecules, a model immunoassay was carried out. In this experiment, mouse IgG was immobilized on the sensing area by using thiol SAM linker molecules and active ester chemistry. A flow-cell was attached to the sensing area in order to flow liquid samples with the affinity binding partner anti-mouse IgG (a-mIgG) that was labeled with a dye Alexa Fluor 670. The fluorescence signal *F* upon sequential flow of samples (PBST) spiked with increasing concentration of a-mIgG was measured in time by using the setup depicted in Fig. 7. Each sample was flowed through the sensor for 10 min followed by the 10 min rinsing. Fig. 13 shows the measured fluorescence kinetics *F*(*t*) for the concentrations of analyte ranging from 30 pM to 30 nM. It reveals that the affinity binding of the labeled analyte to the surface is associated with a gradual increase of the fluorescence signal and that the slope of the fluorescence signal *dF/dt* linearly increases with a-mIgG concentration. The control experiment in which identical samples were injected to the sensor with another antibody immobilized to the gold surface of sensing area (rabbit immunoglobulin G - rIgG) shows a negligible fluorescence signal indicating a highly specific response. From obtained kinetics measured in triplicate, the calibration curve was obtained as shown in Fig. 14. For each analyte concentration, the fluorescence signal slope *dF/dt* in the initial association phase was determined by linear fitting and plotted as a function of the concentration of a-mIgG. The error bars represent the obtained standard deviation (SD) that is attributed to the chip to chip variability. The limit of detection (LOD) of 11 pM was determined at the intersection where the sensor signal *dF/dt* matches 3-fold SD of the baseline fluorescence signal.

CLAIMS:

1. A sensor chip comprising a transparent glass or plastic substrate with at least one concentric grating element and at least one sensing area on its outer surface.

2. The chip according to claim 1, further comprising a metal layer on the sensing area, optionally in the middle of the concentric grating.

3. The chip according to claim 1 or 2, wherein said metal is gold, silver, or aluminum and wherein said metal layer exhibits a thickness between 30 and 70 nm.

4. The chip according to any one of claims 1 to 3, wherein the concentric grating exhibits a relief modulation with the depth between 20-200 nm and wherein said relief modulation is optionally chirped and exhibits the period between 200 nm and 1 μm.

5. The chip according to any one of claims 1 to 4, wherein the concentric grating diffracts surface plasmon-coupled emission beam originating from fluorophores on the sensing area to wave converging at a spot below the chip.

6. The chip according to any one of claims 1 to 5 comprising at least one linear grating that exhibits the modulation depth higher than 10 nm and the period between 200 nm and 1 μm.

7. The chip according to any of claims 1 to 6 in which the linear grating diffracts a monochromatic light beam propagating in a free space to a light beam that propagates in the dielectric substrate and hits the sensing area under an angle of incidence for which surface plasmons are resonantly excited in the sensing area.

8. The chip according to claim 7 in which the linear grating diffracts a monochromatic light beam at the wavelength that is in the absorption band of the fluorophores attached to the sensing area.

9. The chip according to any one of claims 1 to 8, wherein biomolecular recognition elements are attached to the metallic surface, optionally the recognition elements are covalently attached.

10. An apparatus for detecting fluorescence in biochemical assays employing fluorophore labels that combines surface plasmon-enhanced fluorescence and surface plasmon-coupled emission methods, comprising:

a) a chip according to any one of claims 1 to 9; and

b) an excitation source that emits a light beam incident at the linear grating and that is capable exciting fluorophores in the sensing area by surface plasmons; and

c) a light detector arranged to selectively detect fluorescence light that is generated by excited fluorophores, emitted in form of surface plasmon-coupled emission, and imaged to a spot below the chip.

11. The apparatus according to claim 10, comprising the chip as an insertable element, optionally as disposable element, and a permanent reader with a lightsource and a detector.

12. A method for measuring the concentration of an analyte in a sample by combined surface plasmon-enhanced fluorescence and surface plasmon-coupled emission methods comprising the steps of:

a) contacting a sensor chip according to any one of claims 1 to 9 with a sample comprising at least one fluorescence labeled analyte;

b) exposing the fluorescence label at the sensing area to excitation electromagnetic energy in an amount sufficient to achieve surface plasmon-enhanced excitation; and

c) measuring the intensity of surface plasmon-coupled fluorescence emission from fluorophore labels.

13. A flat dielectric substrate with a polymer layer comprising at least one linear grating element and at least one concentric grating element.

14. The polymer layer according to claim 13, wherein said gratings have a depth of at least 10 nm and period between 0.2 and 1 μm.

15. The polymer layer according to claim 13 or 14, wherein said gratings are prepared by using interference lithography, electron beam lithography or nanoimprint lithography.

Fig. 1

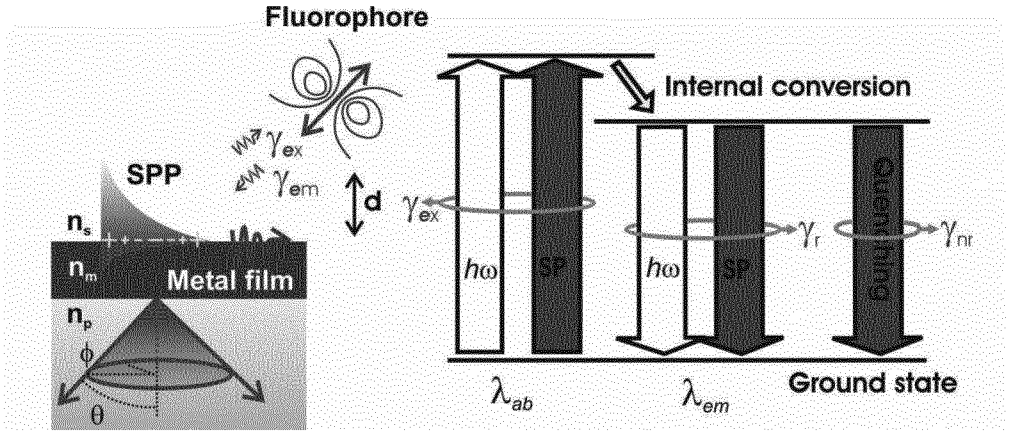

Fluorophore
SPP
n_s
n_m
Metal film
n_p
ϕ
θ
γ_{ex}
γ_{em}
d
Internal conversion
γ_{ex}
$h\omega$
SP
$h\omega$
SP
γ_r
Quenching
γ_{nr}
λ_{ab}
λ_{em}
Ground state

Fig. 2a

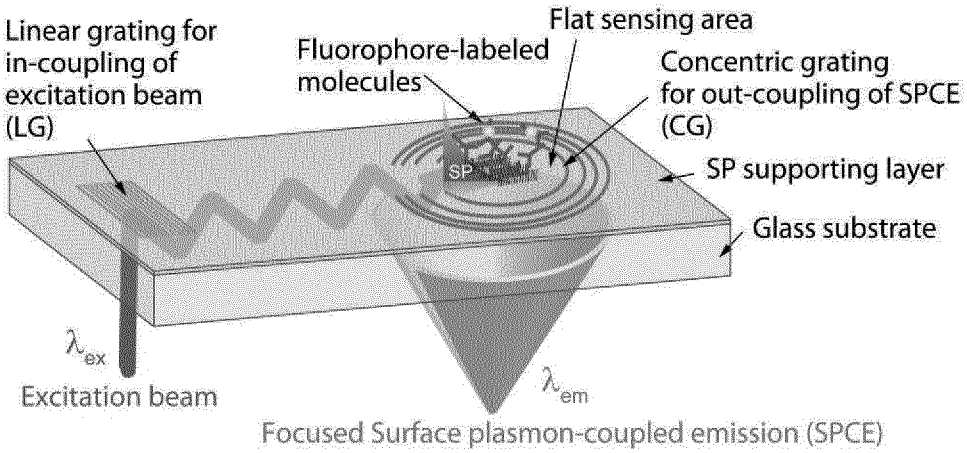

Linear grating for in-coupling of excitation beam (LG)
Fluorophore-labeled molecules
Flat sensing area
Concentric grating for out-coupling of SPCE (CG)
SP supporting layer
Glass substrate
SP
λ_{ex}
Excitation beam
λ_{em}
Focused Surface plasmon-coupled emission (SPCE)

Fig. 2b

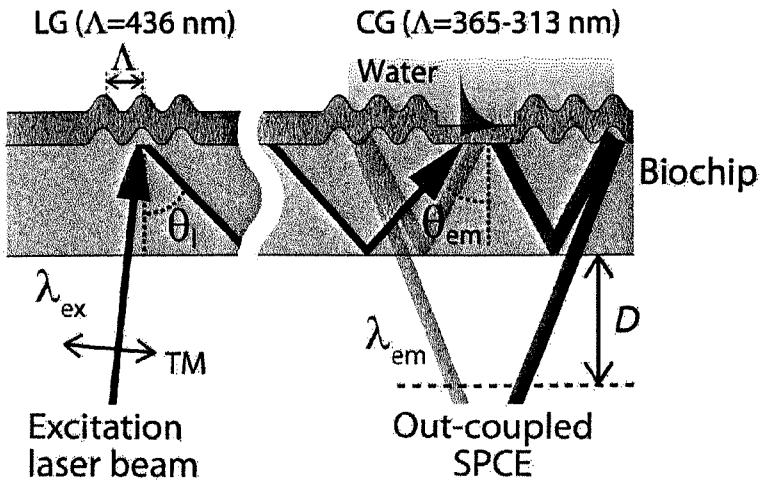

LG (Λ=436 nm)
CG (Λ=365-313 nm)
Λ
Water
Biochip
θi
θem
λex
TM
λem
D
Excitation laser beam
Out-coupled SPCE

Fig. 4

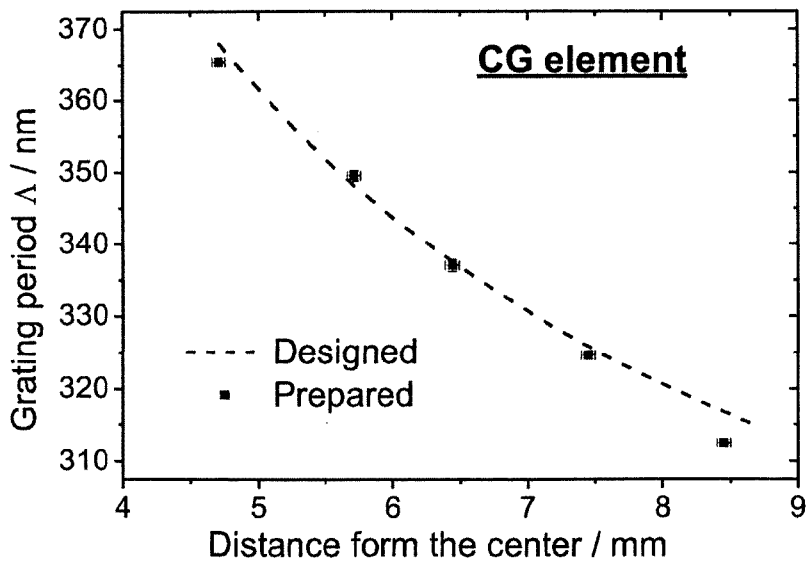

CG element
Grating period Λ / nm
370
360
350
340
330
320
310
4
5
6
7
8
9
Distance form the center / mm
Designed
Prepared

Fig. 3

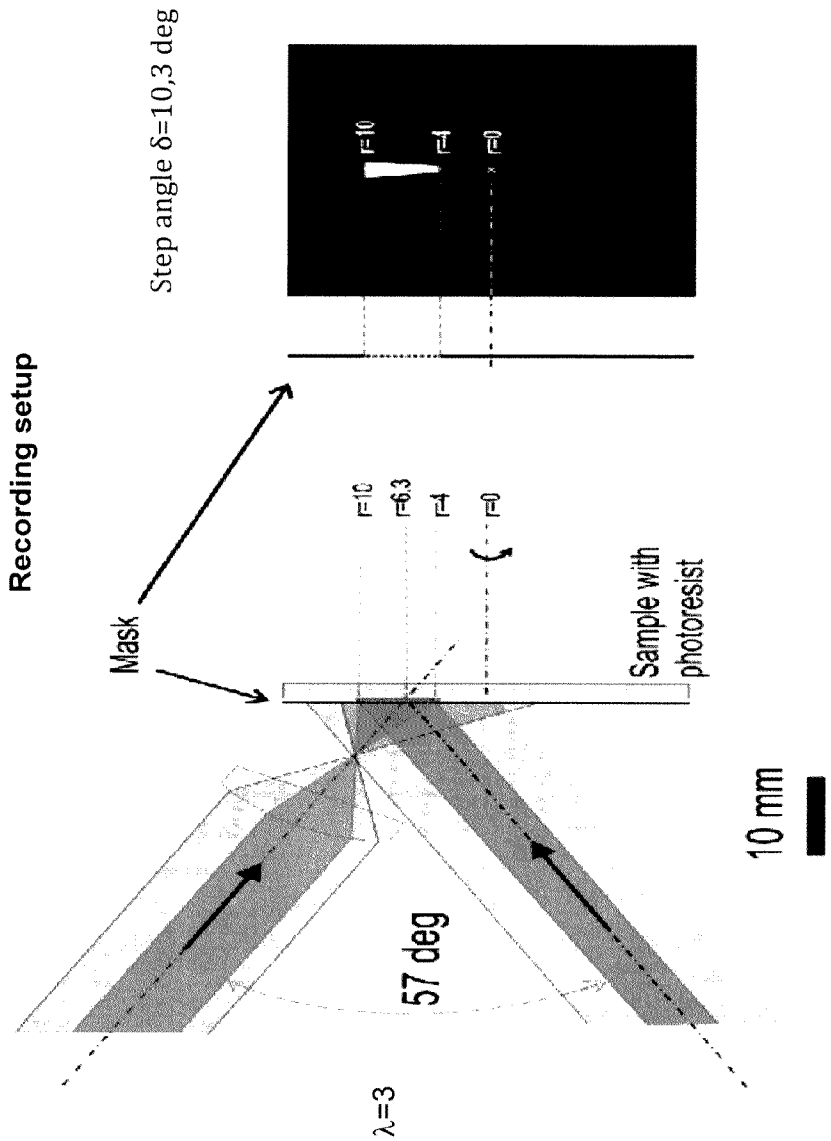

Step angle δ=10,3 deg
r=10
r=4
r=0
Recording setup
r=10
r=6.3
r=4
r=0
Mask
Sample with photoresist
10 mm
57 deg
λ=3

Fig. 5

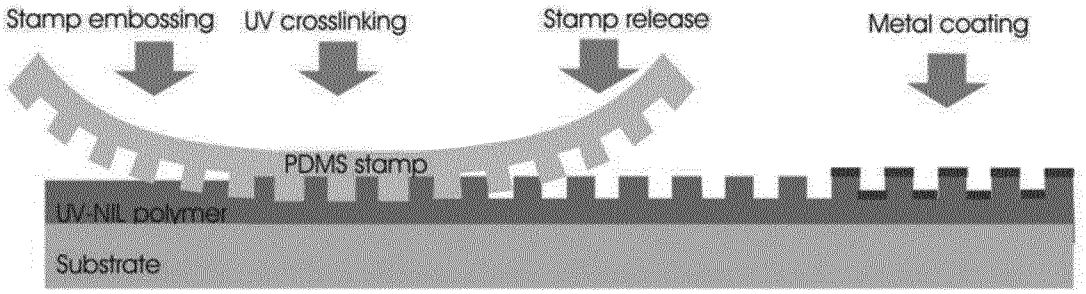

Stamp embossing
UV crosslinking
Stamp release
Metal coating
PDMS stamp
UV-NIL polymer
Substrate

Fig. 6

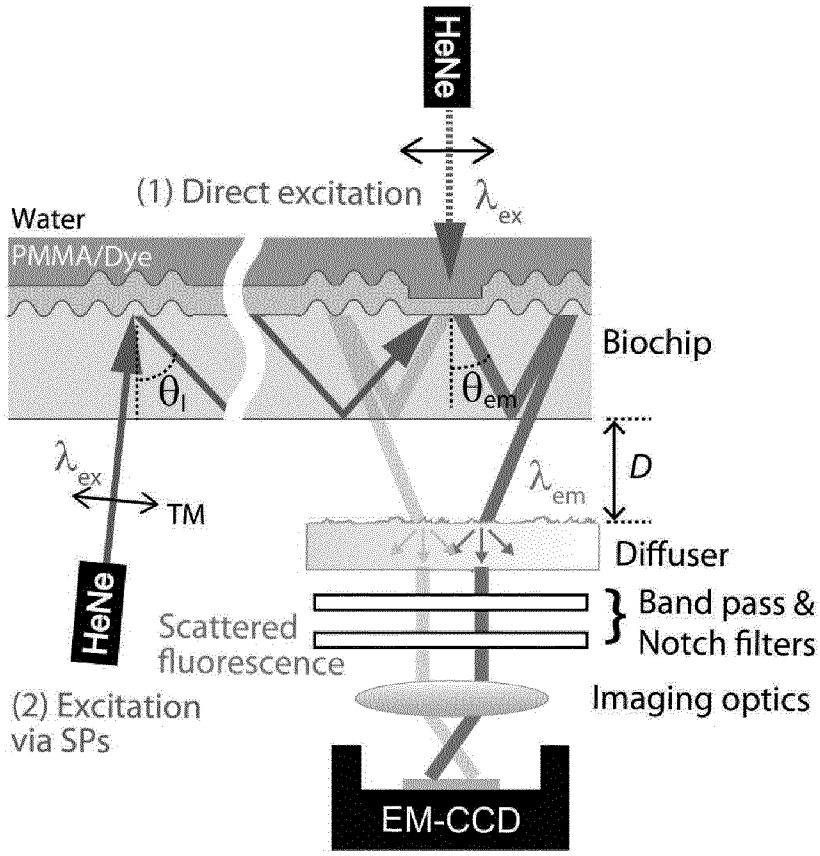

HeNe
(1) Direct excitation
λ_{ex}
Water
PMMA/Dye
Biochip
θ_I
θ_{em}
λ_{ex}
TM
λ_{em}
D
Diffuser
HeNe
Scattered fluorescence
Band pass & Notch filters
(2) Excitation via SPs
Imaging optics
EM-CCD

Fig. 7

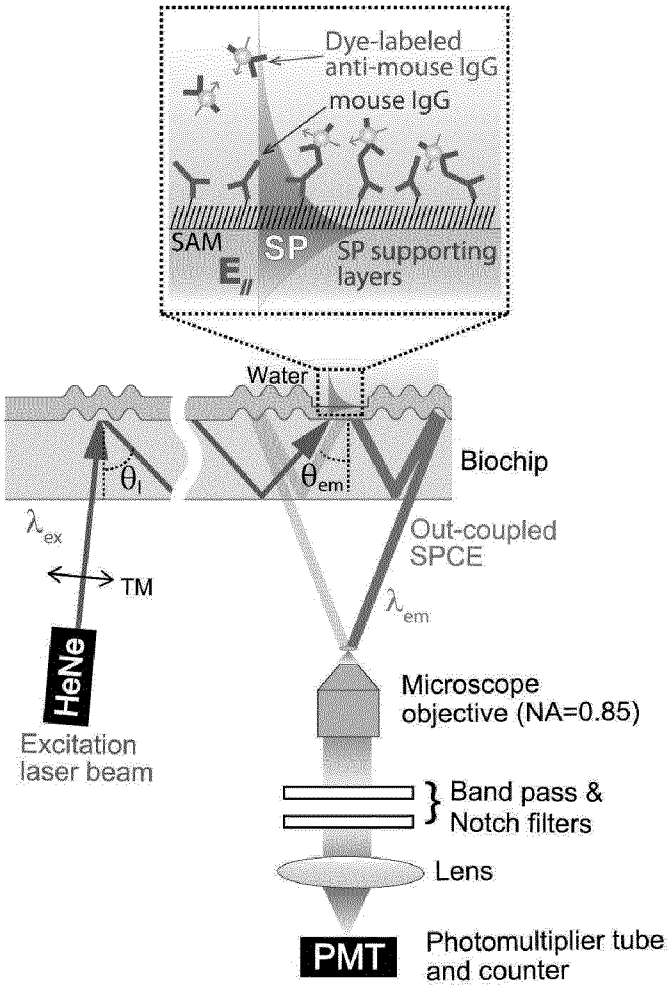

Dye-labeled anti-mouse IgG
mouse IgG
SAM
SP
SP supporting layers
E//
Water
θl
θem
Biochip
λex
TM
HeNe
Excitation laser beam
Out-coupled SPCE
λem
Microscope objective (NA=0.85)
Band pass & Notch filters
Lens
PMT
Photomultiplier tube and counter

Fig. 8

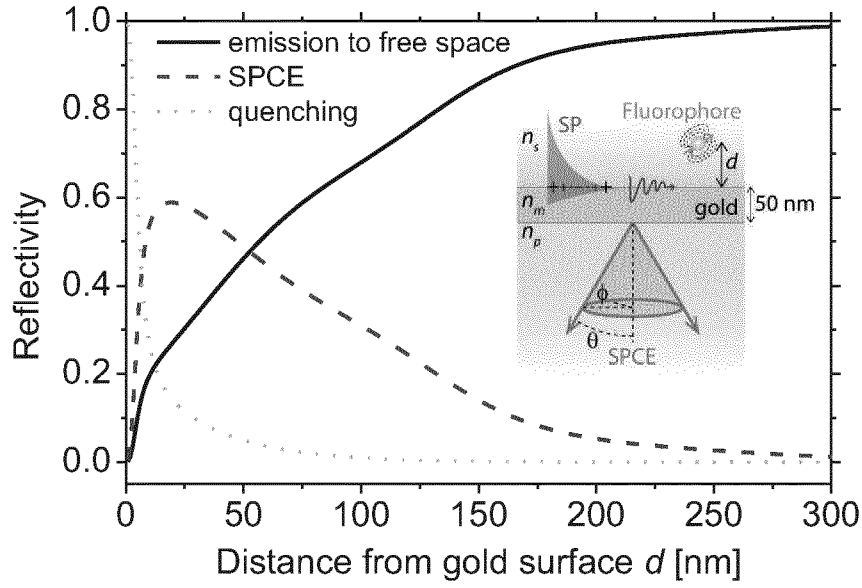

1.0
0.8
0.6
0.4
0.2
0.0
Reflectivity
emission to free space
SPCE
quenching
0
50
100
150
200
250
300
Distance from gold surface d [nm]
SP
Fluorophore
gold
50 nm
SPCE

Fig. 9

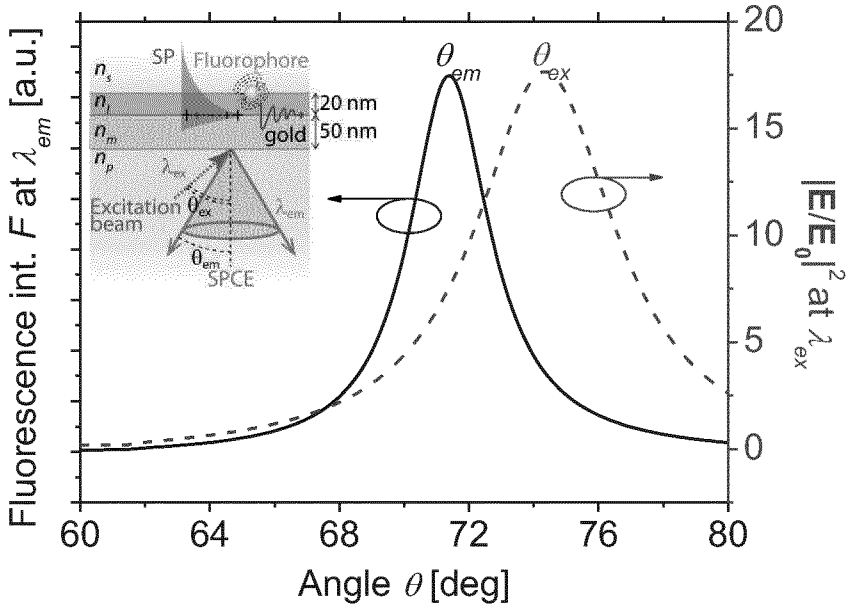

Fluorescence int. F at λem [a.u.]
|E/E0|2 at λex
Angle θ [deg]
θem
θex
SP
Fluorophore
20 nm
50 nm
gold
Excitation beam
SPCE

Fig. 10

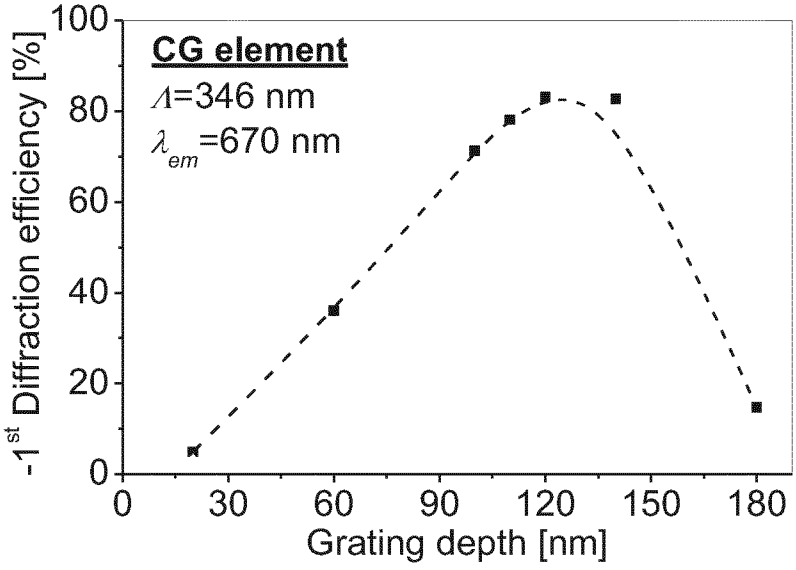

CG element
Λ=346 nm
λem=670 nm
-1st Diffraction efficiency [%]
Grating depth [nm]

Fig. 11

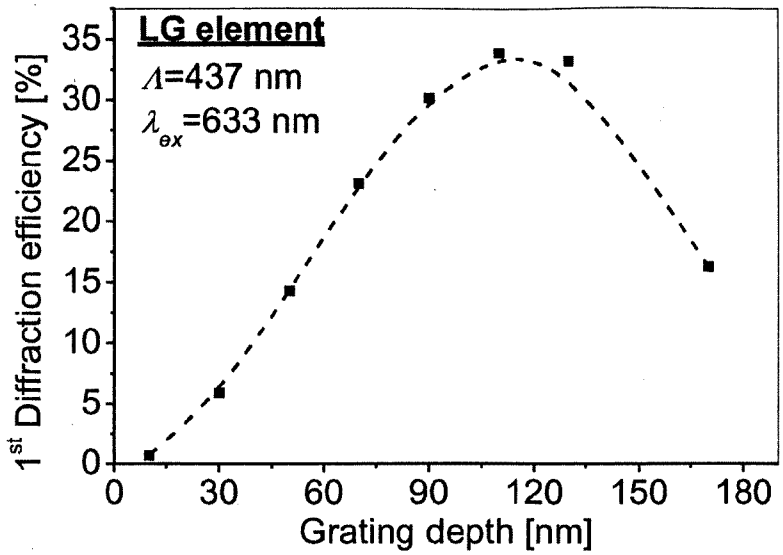

LG element
Λ=437 nm
λex=633 nm
1st Diffraction efficiency [%]
0
5
10
15
20
25
30
35
0
30
60
90
120
150
180
Grating depth [nm]

Fig. 12a

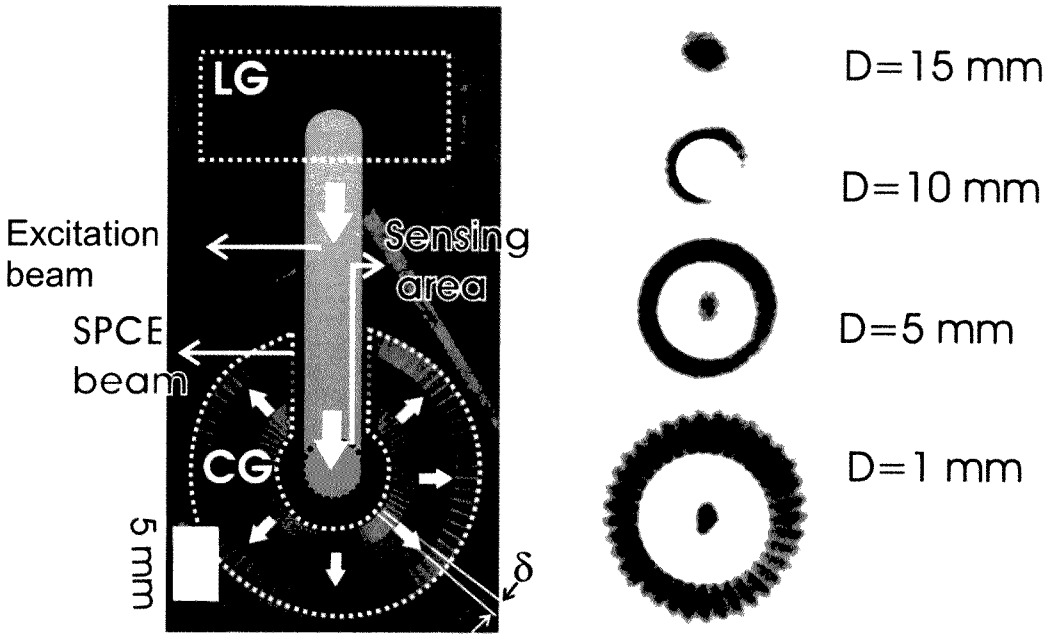

LG
Excitation beam
Sensing area
SPCE beam
CG
5 mm
δ
D=15 mm
D=10 mm
D=5 mm
D=1 mm

Fig 12b

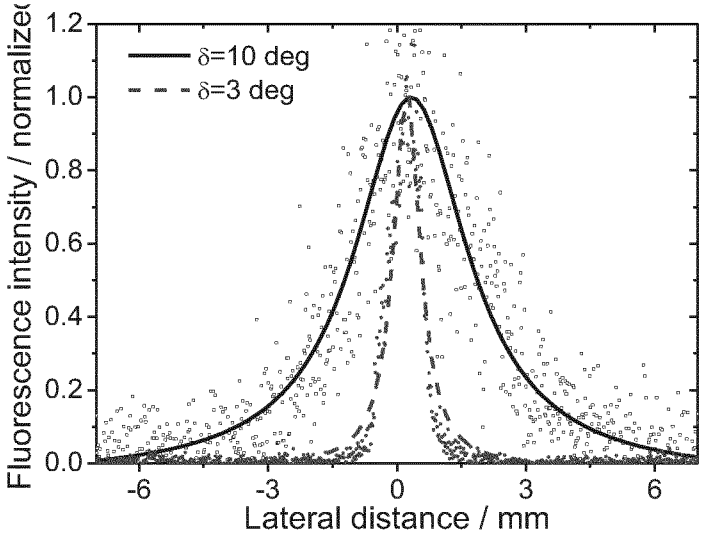


Fig. 13

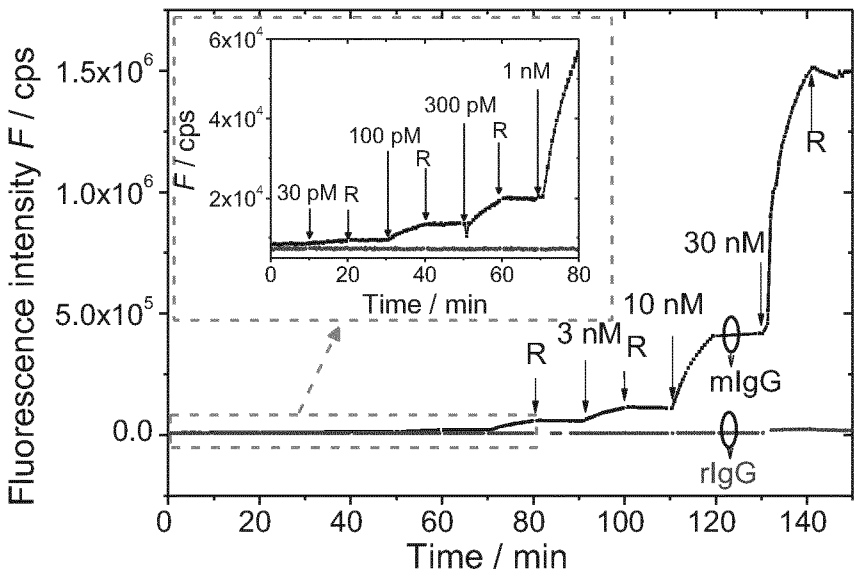
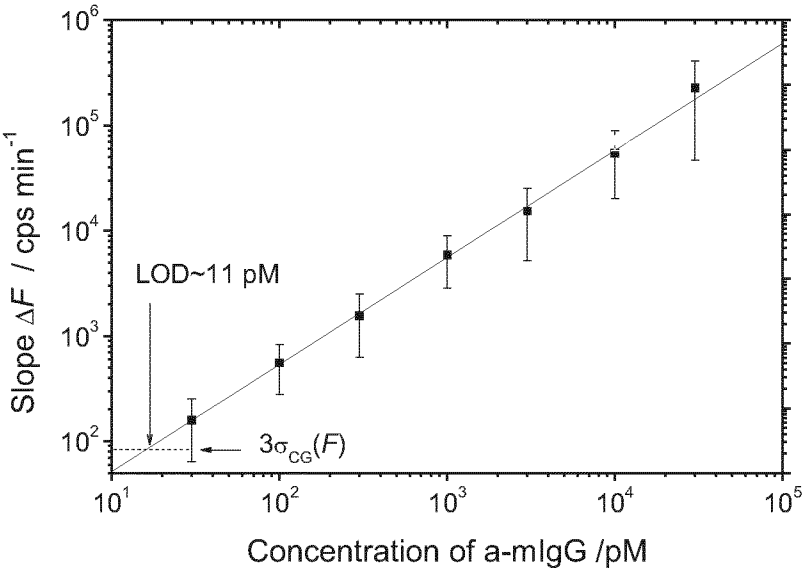


Fig. 14



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/059908

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N21/64 G01N21/77
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N G02B B01L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, INSPEC, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2003/043475 A1 (KATSUMA TOSHIAKI [JP]) 6 March 2003 (2003-03-06) figures 1-4 paragraphs [0068], [0070]	1-12
X	HEYKEL AOUANI ET AL: "Bright Unidirectional Fluorescence Emission of Molecules in a Nanoaperture with Plasmonic Corrugations", NANO LETTERS, vol. 11, no. 2, 9 February 2011 (2011-02-09), pages 637-644, XP055070590, ISSN: 1530-6984, DOI: 10.1021/nl103738d	1-5,9, 11,12
Y	abstract; figures 1-4 page 638, left-hand column, paragraph 1 page 638, right-hand column, paragraph 1 page 640, right-hand column, paragraph 2	6-8,10

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 July 2013	19/07/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Gangl, Martin

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/059908

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/144652 A2 (DELTA DANSK ELEKTRONIK LYS & AKUSTIK [DK]; UNIV DANMARKS TEKNISKE [DK]) 24 November 2011 (2011-11-24)	1,3-5,9, 11
A	abstract; figures 1-9 page 17, line 13 - line 14 page 19, line 27 - page 20, line 11	2,6-8, 10,12
X	GB 2 427 022 A (TOSHIBA RES EUROP LTD [GB]) 13 December 2006 (2006-12-13)	1,2
A	figures 1-18 page 1, paragraph 1 page 12, paragraph 1 page 16, paragraph 7	3-12
Y	JONG SEOL YUK ET AL: "Analysis of immunoarrays using a gold grating-based dual mode surface plasmon-coupled emission (SPCE) sensor chip", THE ANALYST, vol. 137, no. 11, 14 March 2012 (2012-03-14), page 2574, XP055070751, ISSN: 0003-2654, DOI: 10.1039/c2an35143a	6-8,10
A	abstract; figures 1-6 page 2575, left-hand column, paragraph 2 - page 2576, right-hand column, paragraph 3 page 2581, left-hand column, paragraph 3	1-5,9, 11,12
X	US 2012/038918 A1 (LIU ZHIWEN [US] ET AL) 16 February 2012 (2012-02-16) abstract; figures 1-9 paragraphs [0019], [0020], [0035], [0037]	13-15
X	EP 1 644 871 B1 (OVD KINEGRAM AG [CH]) 11 April 2012 (2012-04-11) abstract; figures 1-11 paragraphs [0037], [0092]	13-15
X	US 2006/018021 A1 (TOMKINS DONALD W [US] ET AL) 26 January 2006 (2006-01-26) abstract; figures 1-9 paragraphs [0041], [0046], [0052], [0053], [0064], [0065]	13-15

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/059908

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	KOJI TOMA ET AL: "Compact surface plasmon-enhanced fluorescence biochip", OPTICS EXPRESS, vol. 21, no. 8, 16 April 2013 (2013-04-16) , page 10121, XP055070474, DOI: 10.1364/OE.21.010121 abstract; figures 1-9 page 10124, paragraph 2 - page 10125, paragraph 1 page 10127, paragraph 2 - page 10129, paragraph 1	1-15
A	DARRYN W. UNFRICHT ET AL: "Grating-coupled surface plasmon resonance: A cell and protein microarray platform", PROTEOMICS, vol. 5, no. 17, 13 October 2005 (2005-10-13), pages 4432-4442, XP055070763, ISSN: 1615-9853, DOI: 10.1002/pmic.200401314 abstract; figures 1-9 page 4433, right-hand column, paragraph 2 page 4435, left-hand column, paragraph 1 - page 4437, left-hand column, paragraph 1	1-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2013/059908

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-12

   sensor chip comprising a transparent glass or plastic substrate with at least one concentric grating element and at least one sensing area

   ---

2. claims: 13-15

   flat dielectric substrate with a polymer layer comprising at least one linear grating element and one concentric grating element

   ---

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/059908

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2003043475 A1	06-03-2003	JP 3916200 B2	16-05-2007
		JP 2001272517 A	05-10-2001
		US 2003043475 A1	06-03-2003
WO 2011144652 A2	24-11-2011	EP 2572185 A2	27-03-2013
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EP 1644871 B1	11-04-2012	AT 553449 T	15-04-2012
		AU 2004249864 A1	29-12-2004
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		DE 10328759 A1	20-01-2005
		EP 1644871 A2	12-04-2006
		JP 4550054 B2	22-09-2010
		JP 2007506989 A	22-03-2007
		KR 20060021904 A	08-03-2006
		RU 2344480 C2	20-01-2009
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		WO 2004113953 A2	29-12-2004
US 2006018021 A1	26-01-2006	EP 1771758 A2	11-04-2007
		US 2006018021 A1	26-01-2006
		WO 2006025980 A2	09-03-2006