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(54) Title: CARTRIDGE, DEVICE AND METHOD FOR DETECTING, CAPTURING, IDENTIFYING AND COUNTING CIRCULATING TUMOUR CELLS

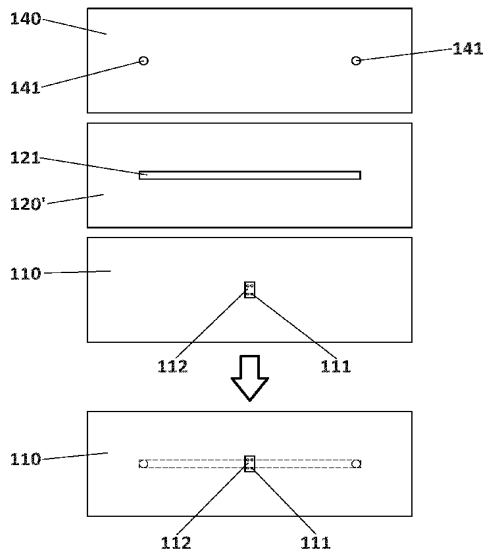


FIG. 6E

(57) Abstract: A cartridge, device and method for detecting, capturing, identifying and counting circulating tumour cells, specifically relating to a cartridge which enables the capture and optionally recovery of circulating tumour cells (CTCs), a device for detecting, identifying and counting CTCs and a method for detecting, identifying and counting CTCs.



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**CARTRIDGE, DEVICE AND METHOD FOR DETECTING, CAPTURING,
IDENTIFYING AND COUNTING CIRCULATING TUMOUR CELLS**

Technical field

5 The present invention relates to a cartridge, device and method for detecting, identifying, capturing and counting circulating tumour cells. Specifically, it relates to a cartridge that enables the capture and optional recovery of live circulating tumour cells (CTCs), a device for detecting, identifying, capturing and counting CTCs and a method for detecting, identifying
10 and counting CTCs.

 The cartridge enables the capture of CTCs from blood samples of cancer patients to subsequently perform an automated count of the number of CTCs captured in said cartridge using an appropriate device. The foregoing is possible due to the execution of a procedure comprising the stages of detecting,
15 identifying, capturing and counting the circulating tumour cells, in addition to the subsequent recovery of said live cells for the genetic analysis of the CTCs.

 The invention is applicable to the sector relating to the detection of cancer progression biomarkers in patients diagnosed with cancer.

20 Background of the invention

 Circulating tumour cells (CTCs) are epithelial cells found in the peripheral blood of cancer patients. The dissemination of CTCs from the primary tumour through the bloodstream gives rise to the development of metastasis, which is the main cause of mortality in cancer. CTC detection has great clinical value,
25 since it enables the early diagnosis of and prognosis for the disease, in addition to monitoring response to treatments. One of the major difficulties of detecting CTCs is their low concentration in the bloodstream. In 1 ml of blood there can be approximately 1 to 10 CTCs per each 10^7 leukocytes and 10^9 erythrocytes, inter alia.

30 Current CTC detection methods are based mainly on the morphological characteristics of the CTCs, on their physical properties (size, density, electric charge) or on immunoseparation techniques. However, separation through the exclusive consideration of physical and morphological properties from blood samples entails sensitivity and specificity problems.

35 Likewise, the application of biosensors based on the Extraordinary

Optical Transmission (EOT) phenomenon is known, which is applied mainly to the detection of molecules and biomolecules smaller than 200 nanometres, but the detection of cells larger than 1 μm has not been achieved to date and much less for the detection of circulating tumour cells (CTCs), whose size varies
5 between 8 and 20 μm .

The devices and methods known in the state of the art do not make it possible to automatically capture, detect, identify, count and recover cells of the aforementioned size and require specialised personnel for each of the phases of the method and particularly for the detection of CTCs, specifically for the final
10 differentiation of the CTC with respect to other types of cells existing in the liquid biopsy carried out on the cancer patient.

As mentioned earlier, the Extraordinary Optical Transmission (EOT) phenomenon in nanostructured metal films, preferably gold, is already known. EOT is an unconventional phenomenon that intensifies the transmission of light
15 across metal surfaces with holes whose size is smaller than the incident light wavelength, in the visible and near-infrared (NIR) region of the electromagnetic spectrum, with a wavelength approximately between 400 nm and 3000 nm. Surfaces with this type of structures are said to be "nanostructured". EOT is caused by the resonant excitation of surface waves (surface plasmons) in the
20 metal due to the coherent oscillation of the electronic plasma induced by electromagnetic radiation. Figures 1a and 1b schematise the phenomenon, such that figure 1a shows the conventional transmission of light through a hole with a radius "r" (where $r > \lambda$), decreasing the intensity of the transmitted light as the incident light wavelength increases. Furthermore, figure 1b shows the
25 extraordinary optical transmission of light through a hole with a radius "r" (where $r \leq \lambda$) in a nanostructured metal film, where the intensity of the transmitted light is maximum for a specific incident light wavelength.

The presence of the surface plasmons makes the EOT phenomenon very sensitive to changes in the optical properties of the support that is in
30 contact with the nanostructured surface. Small variations in these properties give rise to changes in the spectral position of maximum transmission, due to which the nanostructured devices based on this effect can be used as optical sensors of the properties of said support. For this reason, these types of devices are widely used in biomedical applications for detecting minor changes
35 in the composition of biological liquids such as saliva, blood or urine, providing a

useful tool in the early determination of medical diagnoses and, where applicable, the corresponding therapeutic action.

In particular, surface plasmons are inhomogeneous waves that are propagated adhered to the nanostructured surface and have a range of approximately 200 nm from said surface in a perpendicular direction, due to which in order for the device to be sensitive to the changes in the composition of the support, these changes must take place within that range. Most EOT-based devices perform measurements of concentration of proteins or simple molecules, homogeneously distributed and whose size is of nanometric order and, in order to ensure that those molecules are in the sensitive zone, as close as possible to the film, the nanostructured surface is functionalised by means of specific antibodies, such that the nanometric molecules are joined to the antibodies and, therefore, bonded to the surface.

The detection of CTCs in blood from a primary tumour constitutes a biomedical application for detecting biomarkers in biological fluids. The possible colonisation thereof in another area of the body, with the ensuing generation of metastasis which, from a clinical viewpoint, makes the early detection of its presence and the determination of its amount and evolution in the bloodstream of vital therapeutic importance for the clinical oncologist, not only to detect the risk of metastasis, but also to be able to control the effectiveness of a certain treatment in real time.

As opposed to the molecules of nanometric dimensions conventionally detected using EOT-based devices, CTCs have micrometric dimensions (of approximately 10-20 μm), due to which the use of these types of devices for the detection thereof is not trivial nor represents a logical extension of the applications of the EOT phenomenon to the detection of nanometric molecules. This is due to the fact that, as mentioned earlier, the influence of plasmon in a direction perpendicular to the sensory surface reaches approximately 300 nm, due to which objects whose dimensions are smaller than 300 nm can be detected by means of the EOT phenomenon, since once in contact with the sensory surface they remain within the region of influence of the plasmon. However, the manner in which objects whose dimensions exceed 300 nm could be detected, by means of EOT, is not trivial, since once it is in contact with the sensory surface not all the volume of the objects falls within the region of influence of the plasmon. In fact, the samples in which molecules are detected

differ widely from the samples in which CTCs are detected, since the samples with molecules are homogeneous, while the samples with CTCs are inhomogeneous and contain a discrete number of cells, which requires efficient capture on the sensory surface of all the cells.

5 Furthermore, CTC capture using only the functionalisation of the nanostructured surface with specific antibodies and a microfluid channel, integrated in a cartridge, which allows the sample to flow over the nanostructured surface as described in international application WO2015140362-A1, is complicated. Capturing CTCs in this way is generally not
10 very effective and the complication in CTC capture stems from the following causes:

- the very small number of CTCs in blood with respect to any other type of cells, such as lymphocytes,
- CTCs dispersed in a fluid in the interior of a microfluidic circuit are
15 restricted to moving following a laminar flow behaviour, which induces them to pass far from the functionalised surface and, therefore, are not captured with high efficiency,
- there may be cellular components other than CTCs that are unspecifically retained on the biofunctionalized surface, and
20
- heterogeneity in their size, amount and expression of their biomarkers.

In the state of the art no system, device or method is known to provide a solution to the problem of automatically and effectively capturing, detecting, counting and recovering live CTCs from blood samples of patients with metastatic cancer, enabling the analysis of said live cells after recovery. The
25 systems known in the state of the art, such as international publication WO201540362-A1, require passing mononuclear cells from peripheral blood (lymphocytes, monocytes and CTCs) resuspended in a saline buffer through a microfluidic circuit adapted to the surface of a nanostructured sensor, functionalised with antibodies that specifically recognise the CTCs.

30 The document of the state-of-the-art number WO2014/008363-A1 relates to a plasmonic platform for detecting and quantifying different subtypes of HIV. In this case the LSPR effect with metal nanoparticles adhered to this type of virus, with a size of approximately 120 nm and which are present in the blood in an amount significantly greater than that of CTCs found, which facilitates their
35 capture for subsequent detection. Therefore, since the capture of this type of

virus is not a task that entails great difficulty, this document does not provide a detailed description of the type of microfluidic cell to be used to correctly detect the virus. Additionally, in the solution disclosed in this document it is not necessary to guarantee that no virus will escape in the detection, which is vital
5 in the case of detection of CTCs. Likewise, it should be noted that CTCs are 60 times larger than HIV, hence the importance of sample flow and cell design. Therefore, in order to detect CTCs, the design of the microfluidic cell is essential both to guarantee the capture of all the CTCs and to their positioning within the sensory zone and in contact therewith (adhered thereto at a distance
10 smaller than the plasmonic effect). Furthermore, due to the low number of CTCs in the blood, it is indispensable for no false positives to occur, i.e. that no other element which can produce a signal similar to that of the CTC be captured or adhered to the sensory surface of the chip, due to which blocking of the cell surface in a CTC-oriented device is important.

15 Likewise, US patent application number US2012/129192-A1 proposes a system for detecting CTCs by means of a detection method that uses electrical signals generated when the CTCs come into contact with microneedles, said detection method not being based on a photonic system such as that of the present invention. In this document, capture is performed by means of
20 biofunctionalization, taking advantage of the presence of nanoneedles that impede the passage of CTCs, which are trapped therein. It therefore combines biological and mechanical methods regardless of the distance at which the CTCs are trapped with respect to the base of the cell.

Figure 2 exemplifies the theoretical principle of EOT, on which the
25 present invention is based. The light transmitted through the nanostructured sensor has a spectral composition when CTCs are present on its surface with respect to when they are not present. Specifically, figure 2a shows how the periodic presence of nanoholes on a gold surface gives rise to the EOT phenomenon. Figure 2b shows how, after specifically capturing the CTCs with
30 which the surface comes into contact, the spectral composition of the transmitted light changes with the presence of cells captured on the nanostructured surface and the intensity of the transmitted light is maximum for different wavelengths, in accordance with the presence or absence of CTCs, such that the displacement of the position of the spectral transmission peaks
35 indicate the presence of CTCs on the nanostructured surface. Figure 2c

illustrates an experimental result.

The aforementioned differences pose challenges, which have not been addressed to date, in the design of an EOT-based device for detecting CTCs, such as the proximity of CTCs to the sensory surface, the specific capture of
5 CTCs and the detection of CTCs using the spectral position of resonance peaks in different regions of the sensory surface.

Even when the nanostructured surface is specifically functionalised to capture CTCs, it may occur that when performing an optical measurement there are other types of cells on the surface, such as lymphocytes or cell debris,
10 which could give rise to “false positive” type detection errors, i.e. that said cells, lymphocytes or cell debris be considered CTCs.

There exist other documents that refer to biosensing using Localized Surface Plasmon Resonance. Examples of these documents are: Sarah Unser et al, “Localized Surface Plasmon Resonance Biosensing: Current Challenges and Approaches”, *Sensors* 2015, 15, 15684-15716; Mansoureh Z. Mousavi et al, “Label-free detection of rare cell in human blood using gold nano slit surface plasmon resonance”, *Biosensors* 2015, 5, 98-117, and Mansoureh Z. Mousavi et al, “Magnetic nanoparticle-enhanced SPR on gold nanoslits for ultra-sensitive, label-free detection of nucleic acid biomarkers”, *Analyst*, 2013, 138,
15 2740. All these proposals are based on illuminating the whole sensing area and use different strategies in order to enhance the detection sensibility.

EP2653903A1 discloses a plasmonic microscope based on phase measurements, as a consequence of which a phase screen is required. The proposed plasmonic microscope of EP2653903A1 is, however, not suitable for
25 nanostructured chips and biological material.

US2017/0199184A1 discloses a surface coating for capture of circulating rare cells. However, the non-fouling coating disclosed in this document is not suitable for non-reactive surfaces, as are typically metal surfaces, such as gold.

Therefore, a cartridge that integrates a nanostructured surface and a microfluidic channel, which enables the capture of CTCs, in addition to a device
30 that makes it possible to identify and count the CTCs captured by means of the aforementioned Extraordinary Optical Transmission (EOT) with high specificity is necessary, since it is based on the detection of differences in the refractive index between the regions of the nanostructured surface in contact with the
35 CTCs and the regions that could be in contact with the other cellular

components (basically lymphocytes and monocytes). These differences in the refractive index are even more evident in the case of cell debris (basically plasmatic membranes).

5 Description of the invention

The present invention proposes, in accordance with a first object of the invention, a cartridge for capturing and identifying, and optionally recovering CTCs at least 5 μm in size. This cartridge represents a fungible sensor element where the CTCs are captured and optionally recovered live. The cartridge
10 comprises a chip with a nanostructured zone and a coating thereon, and a layer with at least one printed microchannel, disposed on the chip. In use of the cartridge, a fluid having CTCs flows along the at least one microchannel, thus enabling the CTCs to be captured on the nanostructured zone of the chip. The different components of the cartridge are embedded therein.

15 In particular, a cartridge for capturing and label-free identifying circulating tumour cells (CTCs) at least 5 microns in size, is provided. In the context of the present disclosure, "label-free" means that CTCs are identified without using labeled antibodies, such as fluorescently labeled antibodies, that recognize specific proteins on the surface of the CTC. Identification of CTCs with labels,
20 such as fluorescent labels, requires aggressive chemicals that fix the CTCs, in fact dead CTCs, making the extraction of genetic material, such as RNA or DNA, very difficult. In the present disclosure, the optical identification of CTCs manages to maintain CTCs alive and thus, the genetic material (DNA, RNA) is much easier to obtain.

25 The cartridge comprises a chip and a transparent layer disposed on the chip. The chip comprises a transparent substrate, at least one nanostructured zone and a coating on the at least one nanostructured zone. The at least one nanostructured zone has nanoholes distributed on the nanostructured zone with a period of between 500 nm and 900 nm. The nanoholes can have any
30 geometry, such as triangular, square, etc., although they are preferably circular. The nanostructured zone is metallic. The nanostructured zone is at least 20 μm long and at least 300 μm wide. It is disposed on the transparent layer, preferably by means of an adhesion layer. The coating is disposed on the at least one nanostructured zone. The coating is a blocking coating comprising
35 zwitterionic molecules to inhibit attractive chemical interactions with the

nanostructured zone of biological material different to the CTCs, the zwitterionic molecules being self-assembled thus providing homogeneous thickness of the coating. The thickness is comprised within the range of 20 to 150 nm, in order to guarantee that a portion of CTC disposed on the nanostructured zone, is
5 within the most sensitive region of the plasmon excited on the nanostructured zone upon illumination at the required wavelength. In other words, in order to guarantee the detection of the CTC. It is remarked that, taking into account that the nanostructured zone is subjected to optical radiation having wavelength within the spectral ranged comprised in the visible and near-infrared spectrum,
10 a portion of CTC needs to be located at a distance equal to or less than 400 nm with respect to the nanostructured zone, which is the most sensitive region of the plasmon, in order for the CTC to be detected. The selected thickness therefore guarantees that several tens of nm of CTC are within the plasmonic excitation region.

15 The transparent layer disposed on the chip comprises at least one microchannel having a trajectory determined by at least two ends, the at least one microchannel being located on the surface of the at least one nanostructured zone of the chip. The at least one microchannel has a smaller width than the width of the nanostructured zone such that, in use of the
20 cartridge, a fluid comprising CTCs travelling along said at least one microchannel, flows on said at least one nanostructured zone, permitting the CTCs comprised in the fluid to being captured by the nanostructured zone. In other words, the width of the at least one microchannel guarantees that the whole microchannel width is located on the nanostructured zone. The cartridge
25 also comprises holes connected to the ends of the at least one microchannel.

In embodiments of the invention, the homogeneous thickness of the coating is selected to be comprised within the range of 20 to 100 nm.

In embodiments of the invention, the blocking coating with zwitterionic molecules comprises phospholipid bilayers having a double electric charge on
30 the polar head thereof and are anchored to the nanostructured zone by means of thiolated molecules.

In embodiments of the invention, the blocking coating with zwitterionic molecules comprises branched polymers grown using the SI-ATRP technique, based on the adsorption of a polymerisation-initiating thiolated molecule.

35 In embodiments of the invention, the at least one microchannel is

machined on one of the surfaces of the transparent layer. In this case, the depth of the at least one microchannel is smaller than the thickness of the transparent layer. In this case, because the microchannel(s) is(are) not pass-through, the surface opposite the microchannel(s) has holes connected to the ends of the
5 microchannel.

In embodiments of the invention, the at least one microchannel machined on the transparent layer is a pass-through microchannel. In this case, the cartridge further comprises a third layer located on the transparent layer, the third layer having through holes connected to the ends of at least one
10 microchannel. In other words, in this case, because the microchannel(s) is(are) pass-through, an additional layer is required that will be disposed on the second layer (the one having microchannel(s)), covering it. The additional layer may be of a transparent material, such as polycarbonate. It comprises through holes connected to the ends of the at least one microchannel.

In use of the cartridge, the inlet hole or holes are connected to a system that impels a liquid sample through said holes up to the microchannel or microchannels of the cartridge, such that the sample will be located on the nanostructured zone of the cartridge. The blocking coating prevents the material different to the CTCs from interacting with the nanostructured zone and
20 therefore such material flows out of the outlet hole or holes of the cartridge connected to at least one microchannel, while the CTCs remain in the interior of the cartridge. Therefore, material different to the CTCs, that may give rise to false positives, flows out of the cartridge. This is optimised by, in a pre-treatment, adding to the CTCs magnetic nanoparticles functionalised with
25 specific membrane antibodies of the CTCs. Then, in use of the cartridge together with a detection device disclosed next, a magnetic force is applied. The CTCs are thus maintained in the interior of the cartridge and on the nanostructured zone while the liquid sample, typically including material that can give rise to false positives, crosses the cartridge. Because the CTCs are
30 maintained on the nanostructured zone at least for a certain time, CTCs can be detected and counted by means of the detection device.

A second object of the invention is a device for detecting, identifying and counting CTCs. Said device is an instrument comprising an optical system. Using a cartridge, preferably with the characteristics of the cartridge already
35 described, the device is capable of counting the CTCs existing in the cartridge.

Specifically, detection of individual CTCs captured by the cartridge and their identification and quantification is achieved by means of an automated optical configuration of the device including optically scanning the cartridge. Additionally, the device may automatically introduce, in an established volume, the blood sample containing the CTCs captured by the cartridge and recovers them for the subsequent genetic analysis thereof, since said CTCs are still alive because of its label-free identification, that is to say, upon not having been contaminated by external elements that can damage the CTCs.

The device comprises: a support for a cartridge for capturing and label-free identifying CTCs. In order to take full advantage of the device, the cartridge preferably has a chip comprising a nanostructured zone and a coating on the at least one nanostructured zone; and a microchannel enabling the flow on the nanostructured zone of a sample comprising CTCs. The coating is a blocking coating comprising zwitterionic molecules to inhibit attractive chemical interactions with the nanostructured zone of biological material different to the CTCs. The zwitterionic molecules are self-assembled thus providing homogeneous thickness of the coating. The thickness of the coating is comprised within the range of 20 to 150 nm.

The device also comprises an optical illuminating system for, in use of the device, illuminating the cartridge disposed on the support. The optical illumination system comprises a light source and an optical subsystem, the optical subsystem comprising a diaphragm and optical means for providing an image of the diaphragm having a certain illuminating spot on the surface of the chip. The image of the diaphragm is a predefined number of times smaller than the diaphragm. The illuminating spot has a diameter comprised between 10 μm and 50 μm . The device also comprises an optical collector device for collecting the light transmitted through the nanostructured zone of the cartridge. The optical collector device has a transmitted light detection region restricted at least to an area delimited by the illuminating spot. The device also comprises a spectroscopic recording device for recording the transmittance spectra through the cartridge. The spectroscopic recording device comprises a spectrograph for receiving the light transmitted by the optical collector device. The device also comprises a mechanism for the relative displacement between the support for the cartridge and the optical subsystem for enabling the illuminating spot to scan the entire surface of the cartridge; a magnetic mechanism for generating a

uniform magnetic force on the nanostructured zone; a hydraulic mechanism associated with the cartridge for introducing liquid samples in the cartridge and extracting liquid samples therefrom, thus allowing the flow of liquid samples, and a processor for counting the CTCs, controlling, analysing and storing data.

5 A third object of the invention is a method for detecting, label-free identifying and counting CTCs. The method comprises the following stages:

- 10 - Introducing a blood sample in a cartridge having a chip comprising a nanostructured zone and a coating on the at least one nanostructured zone, wherein the coating is a blocking coating comprising zwitterionic molecules to inhibit attractive chemical interactions with the nanostructured zone of biological material different to the CTCs, the zwitterionic molecules being self-assembled thus providing homogeneous thickness of the coating, said thickness being comprised within the range of 20 to 150nm, wherein, prior to
15 introducing the blood sample in the cartridge, the blood sample is prepared using magnetic nanoparticles functionalised with antibodies for capturing CTCs in the sample;
- 20 - Subjecting the cartridge to optical radiation in a certain spectral range, comprised in the visible and near-infrared spectrum, by illuminating the surface of the nanostructured zone of the cartridge with an illuminating spot that is a fraction of the surface of said nanostructured zone, the illuminating spot having a diameter comprised between 10 μm and 50 μm , said optical radiation giving rise to localised surface plasmon resonance (LSPR) in a nanoenvironment near the nanostructured zone of the cartridge;
- 25 - Activation of a magnetic force that acts uniformly on magnetic nanoparticles adhered to the CTCs comprised in the blood sample contained in the cartridge and deposited on the nanostructured zone;
- 30 - Relative displacement between the cartridge support and the illuminating spot throughout the surface of the nanostructured zone of the cartridge;
- 35 - Sequential acquisition of transmittance spectra during the previous displacement, giving rise to a reading of the extraordinary light transmission of light in each of the regions delimited by the illuminating spot, whose sum determines the entire surface of the nanostructured

zone of the cartridge;

- Counting of the CTCs on the nanostructured zone, and
- Extraction of the sample from the cartridge. This may imply the deactivation of the magnetic force. This may also imply the collection of cells in a certain buffer for the subsequent genetic analysis thereof.

5

The introduction into the cartridge and removal of the blood sample from the cartridge is preferably performed by means of a hydraulic system.

In embodiments of the invention, the magnetic force is deactivated prior to the removal of the blood sample.

10

The foregoing cartridge, device and method may give rise to a system which, as a whole, makes it possible to automatically capture, detect, identify, count and optionally recover live circulating tumour cells from blood samples of cancer patients.

15

Therefore, the aim of the invention is to analyse the presence of CTCs in samples of patients obtained from a liquid biopsy, such that the invention automatically integrates the capture, individualised detection of CTCs, counting and optionally subsequent recovery thereof for genetic analysis, with significant CTC capture efficiency and a low probability of nonspecific captures.

20

Due to the foregoing characteristics, the invention is ideal for clinical application in hospital environments.

25

The system is capable of automatically capturing, detecting, identifying, counting and optionally recovering, with high efficiency and specificity, live CTCs from blood samples of cancer patients. This is achieved due to the combined characteristics of the cartridge, such as the presence therein of a nonspecific bonds blocking coating, thereby reducing the cells that remain in the cartridge and reducing "false positive" type errors during detection with the detection device, thereby avoiding, upon reducing said errors, the presence of an expert to determine whether the cell detected by the device is a CTC or other cell, with the characteristics of the device, that makes it possible to perform optical scanning on the entire sensory surface (nanostructured zone) of the cartridge.

30

35

The specificity of the system is increased, as mentioned earlier, due to the use of magnetic nanostructures in the blood samples combined with magnetic mechanisms in the detection device. Specifically, during the preparation of the blood sample prior to its introduction in the cartridge, the

mononuclear cells of peripheral blood (lymphocytes, monocytes and CTCs) are incubated with magnetic nanoparticles functionalised with membrane antibodies specific to the CTCs. Next, the sample with the mononuclear cells resuspended in a saline buffer are passed through the cartridge, specifically, through the microfluidic circuit (microchannels) adapted to the surface of a nanostructured zone, blocked with molecules that reduce the nonspecific bonds and which can be optionally functionalised with antibodies that specifically recognise the CTCs.

Due to the action of the magnetic mechanism of the detection device, this device captures CTCs from the sample and brings them closer to the nanostructured surface of the cartridge, without damaging them, thereby enabling working with live CTCs marked with magnetic nanoparticles, which are susceptible to being specifically attracted only towards the nanostructured zone through the application of a magnetic field restricted to the area delimited by said zone. The magnetic mechanism is preferably a ring-shaped magnet for allowing the light to pass through, having radial magnetisation on the ring plane. Said ring is located underneath the cartridge and specifically underneath the nanostructured area of said cartridge, opposite the layer with a microchannel or microchannels. Another alternative is for the magnet to be a removable opaque magnet that concentrates the magnetic field on the sensory surface. In this case, once the CTCs marked with magnetic nanoparticles have been attracted towards the nanostructured zone, the magnet must be removed to allow the passage of light and perform the detection. In both cases, the magnet is preferably removed to be able to recover the cells, this recovery being optional.

Once the total sample to be examined has passed through the cartridge, particularly after having passed over the chip, the detection device detects the CTCs that have been captured on the nanostructured surface from the spectral position of the resonance peaks in the different regions of the sensory surface. To this end, as mentioned earlier, the detection device performs an action that distinguishes it from any other type of EOT-based device: it optically scans, spot by spot, the entire sensory surface (nanostructured zone), only illuminating at each scanning step one area similar to that typically occupied by an isolated CTC and recording the transmittance spectrum corresponding to each nanostructured region that is illuminated. This action significantly increases the sensitivity of the device and of the system as a whole. The numerical analysis of each of the transmittance spectra makes it

possible to determine the wavelength of the spectral maximum, to subsequently present the results like a binary colour mosaic that identify each of the values of the spectral maximums, and which are geometrically distributed in accordance with the position of the nanostructured surface where they were obtained. The displacement of the spectral transmittance maximum evidences the presence of a change in the refractive index of the medium that is in contact with the nanostructured zone. The choice of colours may be such that it unequivocally shows the position of the CTCs captured on the surface.

Preferably, the stages of the method object of the present invention are implemented by the processor of the device by means of a computer program, such that the processor itself controls the different components of the device which, after correspondingly executing its actions and processing the information recovered, make it possible to obtain as a result, the number of CTCs existing in the cartridge introduced in said device.

In order to exemplify the change in the refractive index that the detection device object of the present application is capable of detecting, it may be started with a CTC having a refractive index of 1.4 immersed in a volume of, for example, $50 \times 50 \times 20 \text{ } \mu\text{m}^3$ of saline buffer having a refractive index of 1.33, and the CTC having a spherical geometry with a radius of $10 \text{ } \mu\text{m}$. The presence of the CTC in said volume converts the refractive index of the medium composed by the buffer and the cell into another, slightly greater, "effective index", which can be determined by means of an elementary calculation, based on approximations of "effective medium". Specifically, it would evolve from a value of 1.33, in the absence of the CTC, to a value of approximately 1.333. That is, the third decimal figure of the index of the buffer without cells would be modified. This change is sufficiently significant to be detected by the detection device and is evidenced, as mentioned earlier, by a spectral displacement of the light transmitted by the nanohole mesh.

In sum, the coating dramatically reduces non-specific bonding of non-CTCs to the nanostructured zone of the cartridge, thanks to which the device mainly reads (detects) CTCs disposed on the nanostructured zone of the cartridge. However, in order to still increase the efficiency of the detection, the differences in the refraction index are used, in order to distinguish, for example, a CTC from a leukocyte.

However, the manufacture of a microfluidic channel with the

necessary dimensions for containing a volume of, for example, $50 \times 50 \times 20 \mu\text{m}^3$ of sample would not be practical for two reasons:

1) an excessively small nanohole mesh would have to be manufactured, i.e. with dimensions of approximately $50 \times 50 \mu\text{m}$, and

5 2) a $50 \times 20 \mu\text{m}$ (width x height) microfluidic channel would have to be designed, which would also be too small and inefficient, due mainly to possible blockings of the channel by the cells of the sample, while requiring an excessively long time to pass from a sample volume of, for example, 2 to 3 ml.

10 These problems are solved by the cartridge object of the present invention, since it uses a chip having nanostructured zone, of for example but not limiting $500 \times 500 \mu\text{m}^2$, and a microfluidic channel of, for a nanostructured zone of $500 \times 500 \mu\text{m}^2$, $1000 \times 500 \times 100$ (length, width, height/depth) microns, allowing the sample to pass without difficulty in short time frames of, for example, less than 4 hours.

15 The advantages of the cartridge, device, method and system object of the present invention are:

- CTC isolation, quantification and optionally recovery actions integrated in a single device compared to independent actions carried out in isolated systems,
- 20 - An increment of the cell detection sensitivity as compared with previous inventions due to the way the system cell+nanostructured surface is illuminated.
- Possibility of implementation at hospital centres for the automated preventive diagnosis of the disease instead of methods based on peoples' experience, carried out in specialised laboratories,
- 25 - The cell is kept alive compared to fixation markers or protocols that kill the cell during counting thereof, such as for example the use of fluorescent markings or permeabilising the cell and causing it to die,
- Automatic detection of independent CTCs compared to detection by
- 30 microscopy equipment that requires interpretation by an expert,
- Blocking that minimizes adherences to the cartridge of unwanted and non-tumour cell lines, minimising the percentage of false positives,
- 35 - If non-specific binding occurs the system distinguishes CTCs from them. In this manner, the system provides the number of CTCs

present in the sample through a software interface, dramatically minimising, or even avoiding, the percentage of false positives.

- Integration of magnetic capture in the device and detection method.

It should be noted that, after obtaining and presenting the results, the live CTCs can be recovered by pumping saline buffer in the cartridge microchannel and recapturing the CTCs marked with magnetic nanoparticles outside of the cartridge. Said CTCs are recovered upon eliminating the magnetic field by moving away from or removing the magnetic mechanism or magnet.

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Brief description of the figures

The foregoing and other advantages and characteristics will be more fully understood from the following detailed description of the embodiments, referring to the attached figures, which must be considered in an illustrative and non-limiting manner, where:

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Figures 1a and 1b show a schematic view of the EOT phenomenon.

Figures 2a, 2b and 2c show a representation of the EOT principle, on which the present invention is based when the light is transmitted through a nanostructured metal film.

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Figure 3 shows an example of a first blocking coating.

Figure 4 shows an example of a second blocking coating.

Figure 5 shows a cartridge object of the present invention.

Figures 6A to 6G show different alternative perspective views of chip configurations which can be embedded in the cartridge. Figures 6A to 6C show an exploded perspective view and two cross-sectional views of a first example of a chip configuration. Figures 6D and 6E show an exploded perspective view and an exploded plan view of a second example of a cartridge. Figures 6F and 6G show an exploded perspective view and an exploded plan view of a third example of a chip configuration.

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Figure 7 shows a schematic view of the device and the position of its components.

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Figure 8 shows an optical assembly of the device of the invention.

Figures 9a and 9b show two possible alternatives of a magnetic mechanism, figure 9a shows an opaque magnetic and removable mechanism and figure 9b shows a ring-shaped magnetic mechanism.

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Figure 10 shows a schematic view of a chip with the fluid passing on it and with a magnetic mechanism located underneath the cartridge.

Figure 11 shows a nanostructured area with CTC cells that incorporate functionalised magnetic nanoparticles.

5 Figure 12 shows a representation of an experimental measurement on a chip of the wavelength shift that occurs in the presence of a cell adhered to the chip and without the presence of a cell on said chip.

Figure 13 shows a possible scanning trajectory by row of the support for the cartridge with respect to the optical device.

10 Figure 14A shows images of the sensory surface of the cartridge and figure 14B shows the diagrammatic representation thereof.

Figure 15A shows images of the result provided by the device and figure 15B shows the diagrammatic representation thereof.

15 Figure 16 shows a schematic view of an alternative device to that of figure 4 and object of the invention.

Figure 17 schematically shows a CTC trapped on the nanostructured zone of a cartridge.

Figure 18 illustrates a plot showing the change in transmittance vs. spectral shift for colorectal tumor cells and leukocytes.

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Detailed description of the preferred embodiment

What follows is a description of a preferred embodiment of the invention referring to the figures that accompany the present description.

25 Figures 5 and 6 show a cartridge 100 for capturing CTCs 300 in accordance with the present invention. When the cartridge 100 is used with a device as for example illustrated in figures 7 and 8, the cartridge is also capable of identifying and counting CTCs. Said cartridge 100 is a fungible product wherein CTCs 300 are firstly captured and subsequently, after having identified the number of CTCs 300 in the cartridge 100, the CTCs 300 may be recovered
30 therefrom.

In figures 6A to 6G several embodiments of chip configurations are shown. The chip configurations may be embedded in the cartridge. Figures 6A to 6G show the substrates or layers that make up the cartridge. The cartridge 100 comprises one chip 110. Figure 10 shows a possible embodiment of the
35 chip 110 comprised in the cartridge 100. The chip 110 is formed by a substrate

130, an adhesive layer 113, a nanostructured zone 112 and a coating 115 disposed on the nanostructured zone 112. The nanostructured zone 112 is metallic. As shown in the figures, the cartridge 100 also comprises a substrate or layer 120, 120' having at least one printed (layer 120') or -engraved- (layer 120) microfluidic microchannel 121, 122. In figures 6D-6G, a third substrate or layer 140 is disposed on the second substrate or layer 120' having at least one printed microfluidic microchannel 121, 122. Layer 120 is disposed on the chip 110 in figures 6A-6C and layers 120', 140 are disposed on the chip 110 in figures 6D-6G. The different substrates or layers, including the one forming the chip 110, are embedded in the cartridge 100 of figure 5.

The dimensions of the chip 110 and in particular of its substrate or layer 130, may coincide with the dimensions of a standard microscope slide. For example, its dimensions may be 75x25 mm (millimetres, 10^{-3} metres). The dimensions of the chip 110 are substantially the same as the dimensions of the layer 120, 120', 140. In fact, the chip 110 and the layer 120, 120' are disposed one over the other, as shown in the figures.

Figure 10 shows a possible embodiment of the chip 110. The chip 110 comprises a substrate 130 and at least one nanostructured metal film 112 disposed on the substrate 130. The nanostructured zone 112 has nanoholes 111, as shown in figures 6A-6G. The substrate 130 is preferably a plastic substrate or a glass substrate, such as pyrex, which is transparent at the working wavelength, i.e. it allows light to pass completely or almost completely. In fact, layers 120, 130 and 140 are transparent in the operating wavelength. The nanostructured zone 112 is made of metal, preferably gold, such as 99.999% gold. The nanostructured zone 112 may be made of a metal other than gold, provided that it fulfils the condition of negative real dielectric permittivity. Non-limiting examples of such metals may be silver, aluminium or any other efficient plasmonic metal. The metal film on which the nanostructured zone 112 is or will be made, may be attached to the substrate 130 by means of an adhesive layer 113, such as a titanium layer. The function of the adhesive layer 113 is to improve the adhesion of the metallic nanostructured zone 112 to the substrate 130, without decreasing light transmission. The adhesive layer 113 may be made of a material other than titanium, such as chrome, which fulfils the condition of facilitating a good adhesion of the metal in contact with the sample.

The thickness of the metal film in the nanostructured zone 112 may vary between 25 and 200 nm (nanometers = 10^{-9} meters), such as between 25 and 100 nm. For example, it may be approximately 50 nm. The thickness of the adhesive layer 113, such as titanium layer, may vary between 1 and 10 nm, such as between 1 and 5 nm. For example, it may be approximately 2.5 nm, not influencing on the transmitted light.

The dimensions of the chip 110 are substantially the same as the dimensions of the substrate 130. The nanostructured zone 112 is preferably limited to an area suitable to receive the total amount of CTCs present in a volume of fluid under inspection. The sides of the nanostructured zone 112 may vary. Its minimum width may be 300 μm and its minimum length may be 20 μm . For example, the area 112 may be 2000 μm x 1000 μm , but these dimensions may vary according to specific applications, conditions, etc. The minimum width of the nanostructured zone 112 is related to the width of the microfluidic channel 121, 122 that runs along the nanostructured zone 112, as detailed below. The minimum length of the nanostructured zone 112 is determined by the size of a single CTCs to be analyzed. For example, a square nanostructured zone of 1 mm per side, or a square sensory film of 500 μm per side, may be used. The sensory film 112 may have other shapes, such as rectangular or circular, provided that its area is suitable to receive the CTCs present in a volume of fluid under inspection. Likewise, the nanostructured zone 112, i.e. the nanohole mesh or matrix or sensory surface is formed by nanoholes 111, such as circular nanoholes 111 of 150-250 nanometres in diameter, although they may have other geometries such as triangular, octagonal, etc., located on the mesh (area) 112 with an interval period of 400 nm to 900 nm, such as 500 nm to 900 nm, preferably 500 nm to 700 nm, more preferably 500 nm to 550 nm. Said period is determined in order for the chip 110 –more precisely, its nanostructured zone 112- to be highly sensitive, while maintaining the spectral position of the EOT resonance within the visible region and the near-infrared spectrum.

Figures 6A to 6C show a first example of embodiment of the cartridge, wherein a printed microchannel 121 is machined on a second substrate 120, also transparent in the wavelength of interest and preferably plastic. When the cartridge is assembled, the second substrate 120, whose dimensions are substantially the same as the dimensions of the chip 110, is attached to the chip 110. The microchannel 121 machined on the second substrate 120 is then

located on the coating 115 of the nanostructured zone 112 and crosses the coating 115 from one side to another, that is to say, the microchannel 121 is disposed along the coating 115 of the nanostructured zone 112. In other words, when a biological sample travels within (along) the microfluidic channel 121, the biological sample gets in contact with the coating 115 of the nanostructured zone 112. The width of the at least one microchannel 121 must be smaller than the width of the nanostructured zone 112. The microchannel 121 has a trajectory determined by two ends. The length of the microchannel 121 may be approximately equal to the length of the chip 110. The microchannel 121 is machined on one of the surfaces of the second substrate 120, such that the depth of the microchannel 121 is smaller than the thickness of the second substrate 120. In other words, in this embodiment, the microchannel 121 does not define a pass-through hole (a pass-through channel, in this case) in the second substrate 120. To access said microchannel 121, holes 126 must be disposed on the second substrate 120, on the surface opposite that of the machining of the microchannel 121, to connect the exterior to said microchannel 121. The components of the cartridge are embedded in such a manner that, in order to connect the cartridge with the exterior, at least one inlet hole 162 and at least one outlet hole 162 for the liquid sample to be introduced in the cartridge, are disposed in the cartridge 100. In particular, the at least one inlet hole 162 and at least one outlet hole 162 are used for introducing the sample with the CTCs 300, and optionally for recovering the CTCs 300 captured on the surface of the chip 110 after the passage of a wash buffer.

The dimensions of the at least one microchannel 121 must be selected taking into account the width of the nanostructured zone 112, the volume of fluid that is going to travel along the microchannel 121 and the speed at which the fluid travels, in use of the cartridge and device of figures 7-8. In other words, the dimensions of the microchannel 121 should allow the fluid traveling therein to do so at an optimum speed while avoiding blockages in the microchannel due to excessively large cells. Therefore, the microchannel dimensions are designed in such a way that a sample having a volume between 1 ml (millilitre, 10^{-3} litre) and 10 ml, such as between 1 ml and 5 ml, is capable of traveling along the microchannel in a reasonable time, such as, a few hours or even minutes. In embodiments of the invention, the length of the microchannel or microchannels is selected to be at least equal to the length of the side of the nanostructured

matrix 111 (that is to say, the length of the side of the sensory film 112 disposed on the chip 110) along which the microchannel is disposed. In embodiments of the invention, the width of the at least one microchannel 121 is selected to be slightly smaller than or equal to the width of the matrix 111 of the sensory film 112, as shown for example in figure 6E. In embodiments of the invention, the length of the at least one microchannel 121 is preferably selected to be at least 500 μm . For example, considering a chip 110 of standard dimensions, such as 75x25 mm, the length of the at least one microchannel may be at least 1 mm, such as at least 2 mm, or at least 3 mm, or at least 4 mm, or at least 5 mm. In embodiments of the invention, the width of the at least one microchannel is selected to be smaller than or equal to the width of the nanostructured zone 112. The minimum width of the microchannel may be 20 μm . In embodiments of the invention, the depth of the at least one microchannel is selected to vary between 50 μm and 150 μm , such as between 70 μm and 130 μm . The second plastic substrate 120 has a dual function: on the one hand, to integrate the microchannels 121 through which the sample circulates in the cartridge 100 and, on the other hand, to serve as a seal, forming a single cell and confining to the microchannel 121 the fluid sample that penetrates the cartridge 100. Although only one microchannel 121 has been represented, the second substrate can include other microchannels with holes connected to the exterior. For example, layer 120 may be selected to be of an adhesive material, such as a pressure-sensitive adhesive (PSA) material.

Figures 6D and 6E show a second example of a chip configuration which, in addition to the chip 110 and second substrate 120', is formed by a third additional substrate 140. In this case, the depth of the microchannel 121 machined in the second substrate 120' is equal to the thickness of said second substrate 120'. In other words, the microchannel 121 is a pass-through microchannel. In this case, the second substrate 120' is preferably a double-sided PSA (Pressure Sensitive Adhesive) film. In order to make the microchannel 121 watertight, a third substrate 140, for example made of polycarbonate, is disposed on said second substrate 120', thus delimiting or sealing the back side of the microchannel with respect to the metal film 112. Said third substrate 140 has pass through holes 141 that connect the exterior of the cartridge to the ends of the microchannel 121. Figure 6E shows a plan view of the independent substrates and a final plan view wherein the different

substrates are superimposed. It can be observed how the width of the microchannel 121 is slightly smaller than the width of the nanostructured 111 sensory film 112.

5 Figures 6F and 6G show a third example of a chip configuration. This example is similar to that of figures 6D and 6E, but it incorporates two microchannels 121, 122. One of the microchannels 121 is similar to the microchannel shown in figures 6D and 6E, while the other microchannel 122 has a branch. In other words, after a first single portion of microchannel, the second microchannel is divided in two branches. On a first end of the third substrate 140 there are two holes 141 that connect the input ends of the two microchannels 121, 122 to the exterior of the cartridge, while on a second end of the third substrate 140 there are three holes 141 that connect the output ends of the microchannels 121, 122 to the exterior of the cartridge. In this example, it can be observed that the chip 110 has three nanostructured zones 112 on which the microchannels 121, 122 of layer 120' are disposed.

15 Therefore, the second substrate 120, 120' can incorporate the microchannel 121 or microchannels 121, 122 in different ways:

- Incorporate microchannels 121, 122 having a depth equal to the thickness of the substrate 120', i.e. the microchannels penetrate and go through the substrate 120', due to which another additional substrate 140 is required to confine the fluid sample, or
- Incorporate machined microchannels 121, 122 on a surface of the second substrate 120, without defining pass through channels, in this case the depth of the microchannel being smaller than the thickness of the substrate 120.

20 To confirm and ensure said confinement, such that the different substrates remain embedded in the cartridge, a base 164 and a lid 163 are preferably used, as shown for example in figure 5. The lid 163 has mechanisms or cams 161 to maintain the base and the lid joined in a waterproof manner. Likewise, the lid comprises connectors 162 that enable the sample to flow into the interior of the cartridge and flow out of said cartridge.

30 Furthermore, the cartridge 100 has a geometry which enables the unique positioning of the cartridge in a device 200, preferably with a tolerance of preferably less than 20 microns to prevent incorrect positioning from affecting the detection and identification action of the device 200. The cooperation of the

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device and the cartridge enables the detection, identification, capture and counting of CTCs 300. Additionally, this geometry prevents the detection device from being used with other cartridges. Additionally, or alternatively to the geometry of the cartridge 100, other elements that guarantee not only the correct position of the cartridge 100 in the detection device 200 but also the exclusive use thereof, can be used. Said elements may be barcodes of the cartridge 100 that identify its compatibility with the device 200, or codes formed by certain geometries, for example, crosses, located at the ends of the cartridge 100, which may coincide with corresponding ones recorded in the device 200 to initiate the reading thereof.

The objective of the aforementioned coating 115, which is disposed on the nanostructured zone 112, is to perform a block with zwitterionic molecules that inhibit attractive chemical interactions with the nanostructured zone 112 of a material different to the CTCs 300, such that they prevent or impede that said cells or biomolecules are trapped by the nanostructured zone 112, thereby reducing the possibility of false positives when detecting and identifying the CTCs 300 in the cartridge 100. The CTCs bind to the nanostructured zone due to the use of biofunctionalized magnetic nanoparticles, as explained later, and a permanent magnet comprised in the device 200, placed just underneath.

The zwitterionic molecules comprised in the coating 115 minimize the binding on non-CTCs, that is to say, biological material different from CTCs, such as leukocytes. In the event that biological material different from CTCs was unwantedly trapped, the surface plasmon created on the nanostructured zone 112 when illuminated with the device that will be described next, makes it possible to specifically distinguish CTCs 300 from any other cellular component that may be retained on the nanostructured zone 112.

As mentioned earlier, the detection of CTCs 300 in blood samples or blood fractions is not easy due to the heterogeneity of the cells present in the sample. The proportion of mononuclear cells, such as lymphocytes and monocytes, with respect to the CTCs 300 in the blood, is in the order of millions. Therefore, the probability of nonspecific bonds between blood cells and biomolecules, such as proteins, on the nanostructured zone 112, is decreased by using a coating having molecules capable of inhibiting attractive chemical interactions with the sensory surface. In this manner, hydrophobic and electrostatic interactions that can take place between the substrate and the

biomolecules present in blood plasma or on the surface of mononuclear cells, are reduced.

The coating of the nanostructured surface 112 stands out for their high density and homogeneity on the entire nanostructured zone 112. This guarantees the scarcity of nonspecific bonds that give rise to “false positive” type errors during the optical scanning performed during the detection process.

The aforementioned coating 115 is adapted to the needs of the principle of detection based on EOT, which requires CTCs 300 to be located at a distance equal to or less than 400 nm with respect to the nanostructured zone 112, which is the most sensitive region of the plasmon. The distance within which the EOT is present depends on the working wavelength. In particular, the maximum distance at which EOT is present is about 400 nm, and this corresponds to a situation in which the nanostructured zone 112 is illuminated with light having a wavelength of around 750 nm. When it is illuminated with light having a different wavelength, the maximum distance at which EOT is present is reduced. For example, at a wavelength of 650 nm, the maximum distance is about 200 nm, that is to say, in order for CTCs to be exposed to EOT, CTCs 300 must to be located at a distance of less than 200 nm from the nanostructured zone 112. In order for the CTCs -to be trapped on the nanostructured zone 112- to be exposed to EOT, the coating 115 is selected to have a homogeneous thickness comprised within the range of 20 to 150 nm, preferably within the range of 20 to 100 nm. Figure 17 shows the nanostructured zone 112 of the cartridge chip 110. The nanostructured zone 112 has been coated with an antifouling coating 115 comprising zwitterionic molecules. As shown in figure 17, a CTC 300 functionalized with magnetic nanoparticles 350 bound to antibodies specific for CTCs, such as anti-EpCAM antibodies, has been trapped on the chip surface. Because the thickness of the coating 115 is lower than or equal to 150 nm, a portion of the trapped CTC is within the plasmonic influence or plasmon depth, which is, in a best scenario, of 400 nm (when the nanostructured zone 112 is illuminated with light having a wavelength of around 750 nm). And when the nanostructured zone 112 is illuminated with light having a different wavelength, such as around 650 nm, in which case the plasmonic influence is of around 200 nm, a portion of the CTC is also within the plasmonic influence. In embodiments of the invention, the cartridge, and therefore its nanostructured zone 112, is illuminated with a

wavelength comprised within the range of 500 to 800 nm. In addition to enabling the optical detection of CTCs because the thickness of the coating 115 is lower than the EOT most sensitive region, the selected thickness of the blocking coating 115 allows magnetic capture of CTCs 300, as will be explained later. In fact, it also allows its chemical modification for the incorporation of more than one type of specific recognition protein biomolecule expressed in the CTC membrane (as different phenotypes appear). This enables the adjustment of the cartridge in accordance with the recognition biomolecules used for different CTC phenotypes, which also implies greater capture specificity and capacity.

This coating 115 comprises zwitterionic molecules, whose net electric charge is zero, but its chemical structure has displacement of positive and negative electrical charges at the operating pH. The separation of the electric charges in the blocking molecules reduces, by means of electrostatic repulsion, the attractive interactions with the cells and biomolecules of the sample. At the same time, the separation of electric charges in the blocking molecules inhibits the hydrophobic interactions between the substrate and proteins that could take place in the support or on the surface of the cells. As said, the coating 115 is a blocking coating comprising zwitterionic molecules to inhibit attractive chemical interactions with the sensory film 112 of biological material different to the CTCs. The coating 115 is fixed to the at least one nanostructured zone 112 of the chip 110. The zwitterionic molecules are self-assembled thus providing homogeneous thickness of the coating 115.

The zwitterionic molecules are applied or deposited on the nanostructured zone 112 by means of auto-assembly techniques, which implies the spontaneous association of molecules based on their chemical composition, through non-covalent bonds into ordered and well-defined molecular aggregates.

In a particular embodiment, the zwitterionic molecules may be phospholipids. The phospholipids are linked to the metal surface by means of the linker molecule. In this embodiment, first, a self-assembled monolayer of a linker molecule, such as MUA (11-mercaptopundecanoic acid), is obtained. The self-assembled monolayer of a linker molecule contains a reactive thiol group for binding to the metal surface (metal nanostructure). The linker is then activated by coupling agents, such as NHS:EDC, that covalently bind to a self-assembled monolayer of phospholipids, such as phosphatidylethanolamide. On

top of this, a second self-assembled layer of phospholipids, such as phosphatidylserine or phosphatidylcholine, is generated, to form a phospholipid bilayer, similar to that of the cell membranes (Figure 3). Phosphatidylethanolamine and phosphatidylcholine have a double electric charge on their polar head and act as zwitterionic molecules. The orientation of the polar heads of the phospholipids towards the sample repels all unwanted cellular interactions. In sum, the lipid bilayers of the coating are supported on the metal film 112 by means of a molecular linker. By using this procedure, the homogeneous distribution and the uniform thickness of the bilayer on the nanostructured zone, is assured, which is required in order to guarantee that a portion of CTC is within the plasmonic influence when the nanostructured zone is illuminated with light causing EOT. In embodiments of the invention, the molecular linker may be thiolated molecules as molecules for anchoring the lipid bilayer to the film.

The molecular linker connects with the metal surface 112 on one side and with the zwitterionic molecules on the other side. The total thickness of the coating 115, including the zwitterionic molecules and the molecular linker, is comprised within the mentioned range of 20 to 150 nm. The molecular linker has two functions: first, it provides groups reactive to the metal, such as gold; second, it enables the formation of a homogeneous layer to join the phospholipids.

It is remarked that phospholipids in aqueous solution tend to aggregate by auto-assembly, but may form different structures, such as in palisade or in micelles, and a uniform and ordered covering of the surface is guaranteed. That is why a linker is used: by adding a linker to the metal surface, self-assembly in palisade is forced and uniform covering on the whole metal surface is guaranteed, which is essential for performing optical measurements. Unlike prior art disclosures, such as US2017/0199184A1, which does not disclose how to bond non-fouling molecules to a scarcely reactive metal, such as gold, which does not generate charge interactions (unlike glass and other polymers, disclosed in US2017/0199184A1, which actually generate charge interactions), the present disclosure has managed to adapt the self-assembly of phospholipids to metal surfaces.

In alternative embodiments of the invention, the blocking coating 115 comprising zwitterionic molecules may comprise polymer brushes (branched

polymers) instead of a phospholipid bilayer, as explained later in accordance with Figure 4. The choice of one or other blocking coating 115 may depend on the type of sample to be analysed.

5 Next, an exemplary method for applying a coating comprising phospholipid bilayers, on a gold film, is disclosed and illustrated in Figure 3. The coating made of phospholipid bilayers is performed on a completely clean gold film immersed in a solution of 2.5mM of 11-mercaptoundecanoic acid (MUA), the MUA being the molecular linker, for 20 hours at room temperature. Afterwards, the surface of the film is washed with ultrapure water and ethanol, 10 and is dried with nitrogen gas. Next, the substrates (coupling agents that activate the linker in order for the phospholipids to be linked) are immersed in a mixture of 1:1 molar solution of NHS:EDC (N-hydroxysuccinimide:N-(3-dimethylaminopropyl)-N-ethylcarbodiimide) for 40 minutes. At the end of the incubation, the gold film is washed with ultrapure water and dried again with 15 nitrogen. Immediately afterwards, the chip (already having the linker) is immersed in a solution of 1 mg/ml of phosphatidylethanolamine (first phospholipid) in chloroform for 1 hour at 4°C, and subsequently washed with chloroform and dried again with nitrogen. Lastly, a preparation of phospholipid with an octylglucopyranoside solution (second phospholipid, to be linked to the 20 first one to form a bilayer) at a certain molar ratio to generate a micellar suspension that is applied to the surface for 2 hours at 50°C was prepared. After this treatment, the micella are adsorbed on the substrate, forming a bilayer, to give the gold film good blocking properties for blocking nonspecific interactions with respect to those of the unmodified gold.

25 Figure 3 shows a schematic view of the stages of formation of the blocking coating of nonspecific attractive interactions made from phospholipid bilayers.

In a second alternative embodiment, illustrated in Figure 4, the coating comprises polymer brushes or branched polymers, preferably grown using the 30 SI-ATRP technique. Auto-assembly techniques, similar to the ones used in the first embodiment, illustrated in Figure 3, are also used. Next, an exemplary method for applying a coating comprising polymer brushes or branched polymers, on a gold film, is disclosed. This technique is based on the adsorption of a polymerisation-initiating thiolated molecule. In other words, the molecular 35 linker used to assemble the polymer brushes or branched polymers with the

metal film 112 is preferably a polymerisation-initiating thiolated molecule. The molecule catalyses a radical polymerisation reaction in the presence of a copper salt solution. This reaction is fed by zwitterionic molecules (shown in Figure 4, linked on one end to the gold film) having a double terminal bond which is activated by the copper catalyst. After adsorption of the initiator, polymerisation takes place in less than 2 hours. This allows the growth of a branched polymer structure as of these monomeric structures. In Figure 4, three different exemplary branched polymer structures have been shown. Figure 4 shows the branched zwitterionic polymers used to form the blocking coating that blocks nonspecific attractive interactions. Polymer brushes include, but are not limited to, poly[N-(2-hydroxypropyl)methacrylamide (pHPMA), poly-sulfobetaine methacrylate (pSBMA) and poly(2-methacryloyloxyethyl phosphorylcholine) (pMPC).

The blocking zwitterionic molecules are preferably derived from phosphatidylcholine, carboxybetaine, sulfobetaine and hydroxyl propylmethacrylamide. This synthesis is carried out under controlled reaction conditions to achieve a high density of blocking polymer and a thickness thereof in accordance with the detection needs based on the EOT phenomenon. These polymers are hygroscopic, which makes them retain water molecules which, in turn, increase the inhibition of hydrophobic interactions that could occur with the proteins of the sample.

Figure 14A shows two images of a surface of the nanostructured zone 112. In the image on the left, a surface 112 without blocking coating is shown, to which a large number of lymphocytes are adhered (in the image they appear as light spots) during the passage of the sample through the chip 110 of the cartridge 110. The presence of these lymphocytes may give rise to false positive results. The image on the right shows a surface 112 with blocking coating to which a low number of lymphocytes is adhered during the passage of the sample through the chip 110 of the cartridge 100. Thanks to the blocking coating, the effectiveness thereof is equal to or greater than 93%, preferably equal to or greater than 96%, which significantly reduces the possibility of obtaining false positive results, because the blocking coating reduces the presence of lymphocytes on the sensory surface 112. Below said two images of figure 14A, figure 14B diagrammatically represents the same images, where the lymphocytes are represented by small circles.

In order to achieve high specificity, the cartridge 100, and particularly the coating applied to the nanostructured zone 112 of the chip 110, is closely related to the detection, identification and counting device 200, in addition to the method for detecting, identifying and counting the CTCs 300 object of the invention, as will be explained next. In other words, the coating dramatically
5 reduces non-specific bonding of non-CTCs, thus reducing the probability of false positives. Thanks to the coating, the device, that will be explained next, is capable of identifying and counting the CTCs trapped on the chip. However, because the coating cannot guarantee 100% efficiency, the optical technique
10 based on differences in refraction index is used, for distinguishing between a CTC and a leukocyte.

As shown in figures 7 and 8, the device 200 for, together with the cartridge 100 already described, detecting, label-free identifying and counting CTCs 300, comprises an automated optical configuration, shown in figure 8,
15 that detects individual CTCs captured by the cartridge 100 and which, after optical scanning of the nanostructured zone of the cartridge where the CTCs are captured, identifies and quantifies them, to subsequently optionally recover the live CTCs for their analysis. That is, the device 200 automatically introduces, in an established volume, the blood sample containing CTCs 300 in
20 the cartridge 100 for it to capture the CTCs and, after the detection, identification and counting, may recover them alive for their subsequent genetic analysis. An alternative diagram of a device is shown in figure 16, in which a power source 250 and a processor with the software or computer program for controlling the device and data analysis, presentation and storage, have been
25 omitted. The system object of the invention, formed by the cartridge and the detection and identification device, is of particular interest for use in hospital environments.

The device 200 comprises a support 220 for receiving the cartridge 100. The device 200 also comprises an illuminating optical system 210, 260 for
30 illuminating the cartridge 100, a light-collecting optical device 231 for collecting the light transmitted by the cartridge, a spectroscopic recording device 230 for recording the transmittance spectra of the cartridge (intensity versus wavelength), a mechanism 270 for performing optical scanning on the cartridge

100, a hydraulic mechanism 240 for introducing and recovering samples in the cartridge 100, a magnetic mechanism 221, 222, preferably a permanent magnet, for generating a magnetic force on the support 220 for the cartridge 100 for capturing CTCs 300, and a processor with the software or computer
5 program for controlling the device and for the data analysis, presentation and storage (not shown). Likewise, the device 200 comprises a power supply 250 for powering the different mechanisms and components, in addition to the necessary computer devices for interacting with the processor and viewing and printing the results of the detection. The scanning mechanism 270,
10 schematically illustrated by means of two arrows in figure 8, is a conventional mechanical displacement device.

The aim of the support 220 for a cartridge 100 integrated in the device 200 is to enable the assembly of a cartridge 100 thereon. Said support 220 and cartridge 100 comprise means for allowing a single positioning of the cartridge
15 on the support. The support 220 has dimensions equal to or greater than those of the cartridge 100. The positioning of the cartridge 100 on the support 220 and, therefore, on the device 200, is critical for the proper functioning of the system formed by the cartridge 100 and the device 200 and, for such purpose, as mentioned earlier, the cartridge 100 has a positioning element integrated
20 therein which enables the device 200 to locate the cartridge in an initial position for the optical scan. In this manner, the device 200 cannot operate any other type of cartridge 100 different from a cartridge having means that are complementary with the support 220.

The optical illuminating system 210, 260 for illuminating the chip 110
25 disposed on the support 220, comprises a light source 210 for providing light through an optical fibre 265 to an optical sub-system 260, in particular to illuminate a diaphragm 262. The optical sub-system 260 comprises at least one optical element 261, a diaphragm 262 and another optical element 264 following the diaphragm 262. Optical element 264 produces an image of the diaphragm 262, also referred to as an illuminating spot, on said chip 110. The
30 optical element 264, is designed to provide an image of the diaphragm 262, the image being a certain (selected) number of times smaller than the diaphragm itself. In a particular embodiment, the optical system or optical element 264 provides an image 10 times smaller than the original diaphragm.
35 The optical element 264 may be implemented by means of an optical

condenser. The element 264 is configured to obtain an image of the diaphragm 262 on the chip surface. In other words, an illuminating spot a number of times smaller than the diaphragm 262 is obtained on the chip surface. The diameter of the illuminating spot (image of the diaphragm) varies between 5 μm and 50 μm , such as between 15 and 30 μm and preferably about 20 μm , thus being a bit bigger than the diameter of a typical CTC. The average size (diameter) of a CTC is typically less than 20 μm .

As a consequence, for example, for an illuminating spot having a diameter of 30 microns, the effective sensitive volume occupied by the CTC, assuming a plasmon penetration of 400nm for a gold nanostructure at lambda 750nm, increases from 0,01% (for a squared chip with side 500 microns) to 3%, i.e. 300 times more. As a consequence, the change in the effective refractive index in the volume of sample illuminated by the illuminating spot of 30 microns, is high enough to be detected by the device of the present disclosure. In contrast, this would never happen with the conventional configurations where a whole chip is illuminated and the effective sensitive volume is much larger. In these cases, the change in the effective refractive index is negligible and consequently, undetectable. The illuminating spot is a fraction of the total surface of the chip 110. In other words, the size of the illuminating spot is selected such that, upon scanning the coated nanostructured zone 110, by displacing the illuminating spot along and across the whole surface of the chip, all the CTCs captured or trapped on the coated nanostructured zone 112, may be counted. The dimension of the illuminating spot can be variable, the diameter of the illuminating spot being a design option, in accordance with the geometric and/or optical differences expected for each specific type of CTCs. The control of the diameter of the spot is carried out acting on the diaphragm 262 of the illuminating system 260 and/or the illuminating system itself. The diaphragm image (or illuminating spot) is formed, through the optical condenser system 264, on the most external surface of the nanostructured zone, that in contact with the sample, buffer+CTCs.

In embodiments of the invention, different light sources 210 may be used. In one possible embodiment, the light source 210 is a broadband white light, such as a halogen lamp, for example a Tungsten halogen lamp. . In another possible embodiment, a light-emitting diode (LED) providing light with a smaller spectral width may be used.

The optical collector device 231 receives the light that is transmitted through the nanostructured zone 112 and guides it up to an optical fibre 232, with the transmitted light detection region restricted to the areas delimited by the variable illumination field. The optical collector device 231 may comprise a
5 collecting lens. As mentioned earlier, said transmitted light detection region is restricted to areas delimited by the illuminator spot, whose preferred diameter is within the mentioned range. The collection of transmitted light, previously
10 focused on the nanostructured zone 112, is performed by means of a microscope objective comprised in the optical collector device 231, for example 10x, 20x or 50x and it is re-focused on the core of an optical fibre 233 for visible-NIR light. This is repeated as many times as the scanning mechanism 270 scans the coated nanostructured metal film of the chip, as explained next.

The spectroscopic recording device 230 for recording the spectral transmittance or EOT of the nanostructured sensing surface, comprises a
15 spectrograph that receives the light guided by the optical fibre 233, i.e. the light transmitted through the cartridge 100 is sent to the spectrograph by means of the optical fibre 232, which is coupled to the inlet of said spectrograph. The spectrograph preferably acquires transmittance spectra in the spectral interval that ranges from 600 nm to 800 nm. As a matter of example, the spectrograph
20 may be configured with an inlet slot with an opening of 300 nm, a spectra acquisition time equal to 0.1 seconds and an accumulation of 60 measurements per spectrum. The spectrograph is controlled by means of the processor of the device 200. It should be noted that the configuration of both nanohole size and period in the chip 110 conditions the spectral transmittance region, meaning
25 that the device 200 is configured to work in a specific region of the spectrum in accordance with a signal from the cartridge 100. Consequently, the components of the device 200 are adjusted in accordance with the characteristics of the cartridge 100. In the case of the use of a LED for illuminating the nanostructured zone, the light transmission for a specific wavelength is
30 recorded and transmittance changes at this wavelength for each illumination spot are studied.

The mechanism 270 for the relative displacement between the support 220 for the cartridge 100 and the optical components 260, which preferably moves the cartridge with respect to the optical means of the device, that remain
35 fixed, makes it possible for the illumination field or illuminating spot to cross

(travel along) the entire surface of the nanostructured zone 110. Said mechanism comes into operation once the entire liquid sample to be examined has passed over the cartridge 100 chip 110 through the microfluidic channel 121, 122, for the device 200 to optically scan the entire nanostructured zone 112 of the chip 110 in buffer, with steps whose size is equal to the dimension of the spot of the optical illumination device 260. The scanning is preferably carried out using piezoelectric motors 270, preferably two, one of the X-axis and another for the Y-axis, controlled by the processor of the device 200, which are coupled to the support 220 for the cartridge 100. These motors 270 make it possible to scan the chip 110 surface in an automated and controlled manner, with nanometric precision. These motors 270 displace the cartridge 100 in such a manner that there are no unscanned areas, i.e. without being illuminated by the light spot, and does so without double readings. To this end, the scan trajectory follows rows, as shown in figure 13, always starting on a first side of the chip 110 to end on the opposite side, the scanning having steps of between 10 and 50 μm , such as 20 μm , the scanning step being the dimension of the illumination spot, and always in the same direction. Once a row has been completed, the scan of the consecutive row begins, also at steps of between 10 and 50 μm , such as 20 μm , and so on, row by row, until the entire chip 110 surface has been covered. In each scan position, the device 200 acquires a spectral reading of the transmitted light and the reading data are processed to obtain comprehensive information of the entire scanned zone or area, as of the data obtained in each area illuminated by the spot.

The device also has a permanent magnetic mechanism 221, 222 placed just under the chip 110 and preferably centred under the nanostructured zone 112. The magnet 221, 222 generates a magnetic force on the magnetic nanoparticles that attract the CTCs 300 on the sensing surface 111-112. As mentioned earlier, one of the critical parameters in a device of these characteristics, intended for capturing cells, is the flow or flow rate through the microchannels 121, 122. The main challenge is to ensure that cells travel at a distance sufficiently close to the nanostructured zone 112 with blocking covering such as to be captured on its surface 112. This is achieved by selecting an appropriate sample flow rate and a suitable permanent magnet that guarantees that the magnetic nanoparticles are under the influence of a strong enough magnetic field to trap the magnetic nanoparticle with its respective

magnet moment onto the nanostructured zone 112.

In order to increase the interaction between the cells of the fluid sample and the nanostructured zone 112, a magnetic capture strategy is used. To this end, the CTC containing sample is previously incubated with magnetic nanoparticles functionalised with antibodies for specific membrane biomarkers of CTCs, such as the anti-EpCAM antibody, or other biomarker antibodies capable of recognizing the different subsets of CTCs including those with mesenchymal or epithelial phenotypic features. In this manner, in the sample introduced in the cartridge 100, only the CTCs 300 have magnetic nanoparticles adhered thereto, as shown in figure 11. Therefore, the CTCs 300 can be attracted towards the nanostructured zone 112 by applying a magnetic field produced by the magnetic mechanism 221, 222 placed underneath the sensory area of the support 220 that supports the cartridge 100, as can be observed in figures 10 and 11. The magnetic mechanism 221, 222 generates a uniform magnetic force on any magnetic material with its respective magnet moment circulating above the nanostructured zone 112.

The dimensions of the magnetic mechanism, such as magnet, are such that they create an intense magnetic field, uniform and perpendicular to the sensory surface of the cartridge 100. To achieve said magnetic field, the following can preferably be used.

- a ring-shaped magnet 222 to allow the passage of light (Figure 9b) and which is radially magnetised along its plane. It is located underneath the sensory surface 112 of the chip 110 and specifically underneath the nanostructured 111 area of said cartridge 100, or
- an opaque magnet that is removable 221 (Figure 9a) that concentrates the magnetic field on the sensory surface 112 of the chip 110, wherein it is necessary to remove the magnet to allow the passage of light and perform the detection.

Said magnetic mechanism 221, 222 is located on the side of the cartridge 100 opposite that of the microchannel or microchannels 121, 122, such that the magnetic force attracts the CTCs towards the nanostructured zone 112. In this manner, the magnetic force on the upper plane of the magnetic mechanism 221, 222 is perpendicular to the surface of the nanostructured matrix, thereby optimising the capture of the magnetic particles

and magnetic particles linked to the captured CTCs 300. In figures 8, 9A and 9B, the magnetic device 221, 222 is disposed between the light-condensing lens 264 and the cartridge 100 support 220, and in figure 16 the magnetic mechanism 221, 222 is disposed between the cartridge 100 support 220 and the light-collecting device 231.

In both cases, the magnet 221, 222 is removed in order to recover the cells once the detection has been performed. This magnetic field enables the CTCs 300 of the fluid sample, which are marked with magnetic nanoparticles 310, to preferably come close to the nanostructured zone 112 while the sample passes through the microfluidic channel 121, 122.

The magnetic field created by the magnet has two functions in the device: firstly, to bring the CTCs 300 closer to the nanostructured zone 112 and secondly, to retain the CTCs 300 in the cartridge 100 during the wash process subsequent to the passage of the sample, wherein all the cells or cell debris that may be nonspecifically adhered are removed, except the aforementioned CTCs 100.

In the event that an opaque magnet is used 221, after washing the cartridge 100 and prior to performing the optical inspection of the chip 110, and with the intention of allowing the passage of light, the magnet is removed 221, since if not, the light would not be able to pass and the reading or detection could not be performed. In order to remove said magnet 221, such as to act or not magnetically on the cartridge 100, it can move either parallel to the cartridge 100 (Y-axis, X-axis) or perpendicular thereto (Z-axis, light beam axis), moving closer to or away from said cartridge 100. In this manner, the CTCs are retained on the sensory surface of the chip and the nonspecific bonds with the surface are significantly reduced. As mentioned earlier, to further reduce the probability of nonspecific bonds, the nanostructured zone can also be functionalised in addition to being blocked by molecules with a double electric charge or zwitterionic molecules. In this manner, during the detection of transmission spectra, the system has a low probability of making "false positive" type errors. The magnetic field of the magnet 221 must be controllable and applicable to the nanostructured zone 112 of the cartridge 100, also using a magnet 221 permanently aligned with the condensing lens 264, wherein the position of the magnet 221 is positioned just underneath the chip 110 of the cartridge 100 secured to the support 220 of the device 200. Said magnet may be for example

manufactured from neodymium, with N50 quality and with a prismatic shape with dimensions of 1x1x3 mm.

Alternatively, and preferably, it is possible to use a ring-shaped magnet 222 radially magnetized that is transparent to light which does not have to be removed to perform the reading and detecting of CTCs 300, since the light passes through it. Subsequently, after having performed the reading and detection, the magnet 222 will also be removed or separated from the cartridge 100 in order to collect the CTCs 300 for the subsequent analysis thereof. The displacement of said ring-shaped magnet 222 is performed in any of the aforementioned ways to displace the opaque magnet 221.

As mentioned earlier, once the cartridge 100 has been introduced in the device support, the sample with the CTCs 300 must be introduced therein and live CTCs 300 must be subsequently recovered for analysis or processed at a laboratory. The foregoing is performed using a hydraulic mechanism 240 linked to said support 220 that makes it possible to introduce liquid samples in the cartridge 100 through the ducts that connect the hydraulic mechanism 240 to the inlet and outlet connectors 162 of the cartridge 100.

The hydraulic mechanism 240 for introducing and optionally recovering samples is such as to allow the sample to pass through the cartridge 100 with a controlled flow without the presence of air bubbles in the interior of said cartridge 100 and for example enabling the injection of wash and removal buffer without contaminating the sample. To this end, as mentioned earlier, the device 200 has a support 220 wherein the cartridge 100 is introduced and the hydraulic mechanism 240 is connected to the injection circuit, and the flow is preferably controlled by a syringe pump. Likewise, it also comprises an "injection loop" that enables the introduction of wash and removal buffer without mixing it with the sample, and also has a degasser that eliminates the presence of air bubbles in the microfluidic channel 121, 122.

As mentioned earlier, another parameter that may be critical in this device intended for cell capture, is the flow or flow rate that circulates through the microchannels 121, 122 of the cartridge 100, since the main challenge is to achieve the displacement of the cells over a distance sufficiently close to the nanostructured zone 112 to be captured thereon. For this reason, the injection pump of the hydraulic mechanism may operate at flow rates of between 1 and 5 $\mu\text{l}/\text{minute}$ for injecting the sample, about 10 $\mu\text{l}/\text{minute}$ to wash the sample and

about 25 $\mu\text{l}/\text{minute}$ to recover the sample. These flow rate values are associated with cell flow speeds that are optimal for passing the sample through the cartridge 100 in the shortest possible time and have the greatest performance in magnetic capture, considering the specific configuration of microchannels 121, 122 of the cartridge 100. This means that the device 200 does not operate properly with cartridges 100 which have a different microchannel 121, 122 configuration.

The last component of the device 200 is a processor for governing the device 200 and the counting of the CTCs 300, in addition to controlling, analysing and storing data. Each of the functions of the device is controlled by proprietary hardware and software of each of the controlled components. The analysis, presentation and storage of data are performed with a computer software or program. In its entirety, the system formed by the device 200 and the cartridge 100 is completely automated, due to which an inexperienced user only has to follow a specific method to make it operate properly and to place the cartridge into the device in the correct way.

In particular, the transmittance spectra obtained for each position of the optical scan are stored in the software and are numerically treated to identify the spectral position of the transmittance peak that is shifted with the presence of CTCs 300 on the nanostructures zone 112 of the chip 110. The software displays the results as a chip 110 map wherein the position of the CTCs 300 that have remained trapped on the chip surface is indicated by means of a colour code. Although ideally, they are only CTCs 300, other types of mononuclear cells (basically lymphocytes) and cell debris may remain in contact with the nanostructures zone 112. The system distinguishes CTCs 300 from nonspecific cells based on the differences in the "effective" refractive index of the illuminated spot. This means that, if a single CTC is compared with a single lymphocyte in one spot, the index differences will arise from the fact that a lymphocyte is smaller than a CTC, due to which the "effective" refractive index of the volume illuminated in the spot will be different. In this manner, the system provides the number of CTCs 300 present in the sample through the software interface, ignoring any other type of cells or debris.

Figure 15A shows two images of the result provided by the device 200 object of the invention. In the image on the left, the superimposition of two images that show the correspondence between the result that is provided by the

device and the real position of cells on the sensory surface can be observed. One of the superimposed images is a photograph in which the presence of three tumour cells (CTCs) on the nanostructures zone 112 and the other superimposed image is the identification, by colour, made by the device 200 of each of the areas of the nanostructures zone 112. It uses a light colour to identify areas with cells and a dark colour to identify areas without cells. In the image on the right the result that the device provides the end user can be observed, which consists of the number of tumour cells (CTC) detected, three, and their position on the nanostructures zone 112 where they were captured. Likewise, figure 15B is included underneath figure 15A wherein the images of figure 15A have been represented diagrammatically, where the three tumour cells can be observed on the diagrammatic image on the left and the identification of the areas of the sensory surface where there is a tumour cell can be observed on the diagrammatic image on the right.

Pursuant to the foregoing, the maximum standard deviation of the spectral displacements detected by the detection device for negative samples is 0.5 nm which, considering the detection limit to be three times that of the standard deviation, means that displacements in excess of 1.5 nm make it possible to detect the presence of CTCs with maximum reliability. Figure 12 shows a graphic representation of an experimental measurement of the displacement in wavelength in a chip with and without a cell.

The foregoing system 200 requires the execution of a method for detecting, identifying and counting CTCs. Said method requires introducing a blood sample in a cartridge 100 with the previously described characteristics and that is the object of the present invention. Prior to introducing the blood sample, it is necessary, as mentioned earlier, to prepare said blood sample for the detection of CTCs 300.

Next, an example of preparation of sample is explained. To prepare the sample to be passed by a cartridge 100, for example between 7 and 10 ml of blood per patient, for example in three 4 ml tubes, are required, preferably of the EDTA type. The fraction of mononuclear cells in which CTCs 300 are included may be obtained using a double-density gradient. To this end, a preparation of polysaccharides, used to separate cells from the blood in accordance with the density of each cell type, for example of the HISTOPAQUE brand, is prepared with a double density gradient, for example in the following

manner: in a Falcon-type 15 ml tube, 2 ml of HISTOPAQUE 1119, with a greater density, are deposited slowly and without touching the tube walls and, on top, 2 ml of HISTOPAQUE 1077 are deposited very slowly, preventing the two phases from becoming mixed. Next, the tube is tilted approximately 45° and 4 ml of blood are deposited very slowly, taking care not to mix the phases. Next, it is centrifuged at 700 g for 30 minutes. After centrifugation, the sample will have become separated in six layers: (1) plasma, (2) a layer of mononuclear cells, platelets and CTCs, (3) HISTOPAQUE, (4) granulocytes and CTCs, (5) HISTOPAQUE and (6) erythrocytes. To recover the greatest number of CTCs, the layer of mononuclear cells and granulocytes is transferred to a 15 ml Falcon tube. Next, the traces of HISTOPAQUE are removed by washing twice with PBS-EDTA 2mM, as follows: 5 ml of PBS-EDTA are added, centrifuged at 700 g for 10 minutes and, lastly, the supernatant is removed. One skilled in the art will understand that some or all of the former preparation steps may be modified in terms of sample volume, time, etc. Immediately afterwards, the cellular pellet (pack of cells remaining at the bottom of the tube after centrifugation) is re-suspended in an amount, such as 500 µl, of magnetic nanoparticles functionalised with the anti-EpCAM antibody for certain time, such as 30 minutes, at a temperature of, for example, 4°C. The preparation procedure can be used with variations in the values of the components of up to around 10%.

Next, the sample obtained from the three tubes is joined and introduced in the cartridge 100 through the hydraulic mechanism 240 of the device 200, in order to let CTCs be trapped by the nanostructured zone of the cartridge and, upon operation of the device, be counted. The sample may be introduced by placing the prepared sample in a 1 ml disposable syringe. The syringe is introduced in the syringe pump of the device and its tip is connected to the tube 242 connected to the cartridge 100. Furthermore, a PBS (Phosphate Buffered Saline) buffer or other physiological saline buffers may be placed in the injection "loop" of the device. The "loop" valve is placed in the position that enables the passage of the PBS and the syringe pump is activated with a flow rate of, for example, between 5 and 50 µl/minute, to remove any air bubble that there could be in the cartridge 100. In other words, the sample having CTCs is inserted in the device in order for them to travel through the cartridge. After certain time, such as 2 minutes the operation of the pump is stopped, the "loop" valve is placed in the position that enables the passage of the sample and the syringe

pump is reactivated with a flow rate between 5 and 50 $\mu\text{l}/\text{minute}$. The pump remains operational until the syringe sample has become exhausted. Subsequently, the "loop" valve is placed in a position that enables the passage of the PBS to wash the cartridge 100 and remove all the components nonspecifically bonded to the sensory surface 112.

The foregoing is performed while a magnetic force of the opaque magnet 221 or ring-shaped magnet 222 is applied, which acts on the blood sample contained in the cartridge 100.

Once the CTCs are trapped in the cartridge disposed on the support of the device, in order to illuminate them in turn for identifying and counting them, if an opaque magnet is used, it must be removed prior to illumination. If, on the contrary, a ring-shaped magnet is used, there is no need to remove it prior to illuminating the cartridge. In the case of an opaque magnet 221 and in order to remove it, it must be moved away from the cartridge 100, due to which it is possible to proceed by firstly moving away the condensing lens 264 of the device 200; next, the magnet 221 is displaced from the vertical position and, lastly, the condensing lens 264 is moved closer until the field diaphragm 262 is focused.

After having removed the magnetic field, the cartridge 100 is subjected to optical radiation in a certain spectral range in the visible and near-infrared spectrum by illuminating the surface of the sensory film 112 of the chip 110 with an illumination field which is, as mentioned earlier, a fraction of the surface of said sensory film 112, producing a surface plasmon on the nanostructure 111 of the cartridge 100 chip 110. In the case that a ring-shaped magnet 222 is used, it is not necessary to remove it to illuminate the sensory surface 112 of the chip 110 or cartridge 100. Said illumination is performed through the central hole of the magnet 222.

This is followed by the relative displacement between the cartridge 100 support 220 and the illumination field throughout the surface of the sensory film 112, for which purpose the motorised 270 cartridge 100 support 220 is preferably displaced to a position where the surface of the nanostructured 111 sensory film 112 is not illuminated. Once there, the "acquire background" option is selected in the computer program or software and when the software indicates that the process has finished, it enables the passage of light to the optical fibre by means of a switch incorporated in the device 200 and the

“acquire reference” option is selected in the computer software or program.

Once the software indicates that it has acquired the reference, it is placed, with the motorised 270 cartridge 100 support 220, in the position in which the field diaphragm 262 illuminates, for example, only the lower right-hand corner of the nanostructured 111 area, after which the computer software or program begins to scan the cartridge 100 (it could begin with any of the four corners). This is performed to obtain the reference and also to obtain the measurement with blood sample in exactly the same area. Next, the difference is measured in each part of the nanostructured 111 area following the same sequence of movement. Once the optic scanning has been completed, the software informs that the analysis of the results has been carried out and displays them on a screen on the computer, indicating the number of CTCs 300 present in the sample. During said scan or displacement, a sequential acquisition of spectra takes place, giving rise to an extraordinary light transmission reading in each of the regions delimited by the illumination field, and whose sum determines the entire cartridge 100 chip 110 surface.

After said scan, the spectra acquired are analysed to count the CTCs 300 existing in the cartridge 100.

Lastly, in a particular embodiment, in order to recover the live CTCs captured by the cartridge, the opaque magnet, if used, is removed and the recipient connected to the outlet of the hydraulic pumping mechanism 240 is changed and buffer is injected at a flow rate of between 25 and 100 $\mu\text{l}/\text{minute}$ to recover the live CTCs 300

Because the cartridge of the invention, in particular its nanostructured zone, does not need to be functionalised using specific antibodies, such as anti-EpCAM antibodies, the cartridge and device enable label-free identification, thus preventing damage of the CTCs.

Figure 18 illustrates a plot showing the results of an experiment which proves that the cartridge and device of the present disclosure permit to distinguish between CTCs and leukocytes. In particular, figure 18 shows the change in transmittance vs. spectral shift for colorectal tumor cells and leukocytes.

The foregoing example is one of the possible embodiments of the invention object of the present application and must not be considered to be limiting.

CLAIMS

1. A device (200) for detecting, identifying and counting CTCs, characterised in that it comprises:
- 5 a support (220) for a cartridge (100) for capturing and label-free identifying CTCs, the cartridge (100) having a chip (110) comprising a nanostructured zone (112) and a coating (115) on the at least one nanostructured zone (112), wherein the coating (115) is a blocking coating comprising zwitterionic molecules to inhibit attractive chemical interactions with
- 10 the nanostructured zone (112) of biological material different to the CTCs, the zwitterionic molecules being self-assembled thus providing homogeneous thickness of the coating (115), said thickness being comprised within the range of 20 to 150nm,
- an optical illuminating system (210, 260) for, in use of the device (200),
- 15 illuminating the cartridge (100) disposed on said support (220), the optical illumination system (210, 260) comprising a light source (210) and an optical subsystem (260), the light source (210) being configured to provide optical radiation in a certain spectral range comprised in the visible and near-infrared spectrum, the optical subsystem (260) comprising a diaphragm (262) and
- 20 optical means (264) for providing an image of the diaphragm (262) having a certain illuminating spot on the surface of the chip (110), the image of the diaphragm (262) being a predefined number of times smaller than the diaphragm (262), said illuminating spot having a diameter comprised between 10 μm and 50 μm ,
- 25 an optical collector device (231) for collecting the light transmitted through the nanostructured zone (112), the optical collector device (231) having a transmitted light detection region restricted at least to an area delimited by the illuminating spot,
- a spectroscopic recording device (230) for recording the transmittance
- 30 spectra through the cartridge, which comprises a spectrograph for receiving the light transmitted by the optical collector device (231),
- a mechanism (270) for the relative displacement between the support (220) for the cartridge (100) and the optical subsystem (260) for enabling the illuminating spot to scan the entire surface of the cartridge (100),
- 35 a magnetic mechanism (221, 222) for generating a uniform magnetic

force on the nanostructured zone (112),

a hydraulic mechanism (240) associated with the cartridge (100) for introducing liquid samples in the cartridge (100) and extracting liquid samples therefrom, thus allowing the flow of liquid samples, and

5 a processor for counting the CTCs, controlling, analysing and storing data.

2. The device, according to claim 1, characterised in that the magnetic mechanism is a permanent magnet (221, 222).

10

3. The device, according to any of claims 1 or 2, characterised in that the spectroscopic recording device (230) acquires transmittance spectra in the spectral interval corresponding to the visible and near-infrared spectrum.

15 4. The device according to any preceding claim, wherein the size of the illuminating spot is a fraction of the total surface of the nanostructured zone (112).

20 5. The device according to any preceding claim, wherein optical means (264) for providing an image of the diaphragm (262) having a certain illuminating spot on the surface of the chip (110), is an optical condenser.

25 6. The device according to any preceding claim, wherein the cartridge (100) to be disposed in said support (220) is a cartridge (100) for capturing and label-free identifying circulating tumour cells (CTCs) at least 5 microns in size, which comprises a chip (110) and a transparent layer (120, 120') disposed on the chip (110), the chip (110) comprising:

a transparent substrate (130),

30 at least one nanostructured zone (112) having nanoholes (111) distributed on the nanostructured zone (112) with a period of between 500 nm and 900 nm, the nanostructured zone (112) being metallic, the nanostructured zone (112) being at least 20 μm long and at least 300 μm wide, the nanostructured zone (112) being disposed on the transparent substrate (130) by means of an adhesion layer (113),

35 a coating (115) disposed on the at least one nanostructured zone (112),

wherein the coating (115) is a blocking coating comprising zwitterionic molecules to inhibit attractive chemical interactions with the nanostructured zone (112) of biological material different to the CTCs, the zwitterionic molecules being self-assembled thus providing homogeneous thickness of the coating (115), said thickness being comprised within the range of 20 to 150nm, and a transparent layer (120, 120') disposed on the chip (110) comprising at least one microchannel (121, 122) having a trajectory determined by at least two ends, the at least one microchannel (121, 122) being located on the surface of the at least one nanostructured zone (112) of the chip (110), the at least one microchannel having a smaller width than the width of the nanostructured zone (112) such that, in use of the cartridge (100), a fluid comprising CTCs travelling along said at least one microchannel (121, 122), flows on said at least one nanostructured zone (112), permitting the CTCs comprised in the fluid to being captured by the nanostructured zone (112), the cartridge comprising holes (126, 141, 162) connected to the ends of the at least one microchannel (121, 122).

7. A cartridge (100) for capturing and label-free identifying circulating tumour cells (CTCs) at least 5 microns in size, which comprises:

a chip (110) and

a transparent layer (120, 120') disposed on the chip (110),

the chip (110) comprising:

a transparent substrate (130),

at least one nanostructured zone (112) having nanoholes (111) distributed on the nanostructured zone (112) with a period of between 500 nm and 900 nm, the nanostructured zone (112) being metallic, the nanostructured zone (112) being at least 20 μm long and at least 300 μm wide, the nanostructured zone (112) being disposed on the transparent substrate (130) by means of an adhesion layer (113),

a coating (115) disposed on the at least one nanostructured zone (112), wherein the coating (115) is a blocking coating comprising zwitterionic molecules to inhibit attractive chemical interactions with the nanostructured zone (112) of biological material different to the CTCs,

the zwitterionic molecules being self-assembled thus providing homogeneous thickness of the coating (115), said thickness being comprised within the range of 20 to 150nm, and a transparent layer (120, 120') disposed on the chip (110) comprising at least one microchannel (121, 122) having a trajectory determined by at least two ends, the at least one microchannel (121, 122) being located on the surface of the at least one nanostructured zone (112) of the chip (110), the at least one microchannel having a smaller width than the width of the nanostructured zone (112) such that, in use of the cartridge (100), a fluid comprising CTCs travelling along said at least one microchannel (121, 122), flows on said at least one nanostructured zone (112), permitting the CTCs comprised in the fluid to being captured by the nanostructured zone (112), the cartridge comprising holes (126, 141, 162) connected to the ends of the at least one microchannel (121, 122).

15

8. The cartridge, according to claim 7, wherein the homogeneous thickness of the coating (115) is comprised within the range of 20 to 100 nm.

9. The cartridge, according to either claim 7 or 8, characterised in that the blocking coating (115) with zwitterionic molecules comprises phospholipid bilayers having a double electric charge on the polar head thereof and are anchored to the nanostructured zone (112) by means of thiolated molecules.

10. The cartridge, according to any of claim 7 or 8, characterised in that the blocking coating (115) with zwitterionic molecules comprises branched polymers grown using the SI-ATRP technique, based on the adsorption of a polymerisation-initiating thiolated molecule.

11. The cartridge, according to any of claims 7 to 10, wherein the at least one microchannel (121, 122) is machined on one of the surfaces of the transparent layer (120), the depth of the at least one microchannel (121, 122) being smaller than the thickness of the transparent layer (120).

12. The cartridge, according to any of claims 7 to 10, wherein the at least one microchannel (121) machined on the transparent layer (120') is a pass-

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through microchannel, the cartridge further comprising a third layer (140) located on the transparent layer (120'), the third layer (140) having through holes (141) connected to the ends of at least one microchannel (121).

- 5 13. A method for detecting, label-free identifying and counting CTCs, characterised in that it comprises the following stages:
- 10 - Introducing a blood sample in a cartridge (100), the cartridge (100) having a chip (110) comprising a nanostructured zone (112) and a coating (115) on the at least one nanostructured zone (112), wherein the coating (115) is a blocking coating comprising zwitterionic molecules to inhibit attractive chemical interactions with the nanostructured zone (112) of biological material different to the CTCs, the zwitterionic molecules being self-assembled thus providing homogeneous thickness of the coating (115), said thickness being comprised within the range of 20 to 150nm, wherein, prior to introducing the blood sample in the cartridge (100), the blood sample is prepared using magnetic nanoparticles (350) functionalised with antibodies for capturing CTCs in the sample,
 - 15 - Subjecting the cartridge (100) to optical radiation in a certain spectral range comprised in the visible and near-infrared spectrum, by illuminating the surface of the nanostructured zone (112) of the cartridge (100) with an illuminating spot that is a fraction of the surface of said nanostructured zone (112), the illuminating spot having a diameter comprised between 10 μm and 50 μm , said optical radiation giving rise to localised surface plasmon resonance (LSPR) in the nanoenvironment near the nanostructured zone (112) of the cartridge (100),
 - 20 - Activation of a magnetic force that acts on the blood sample contained in the cartridge (100),
 - Relative displacement between the cartridge (100) support (220) and the illuminating spot throughout the surface of the nanostructured zone (112) of the cartridge (100),
 - 25 - Sequential acquisition of transmittance spectra during the foregoing displacement, giving rise to a reading of extraordinary light transmission in each of the regions delimited by the illuminating spot, whose sum determines the entire surface of the nanostructured zone (112) of the cartridge (100),
 - 30 - Counting of the CTCs on the nanostructured zone (112) of the cartridge
 - 35

(100), and

- Removal of the sample from the cartridge (100).

5 14. The method according to claim 13, characterised in that the introduction into and removal of the blood sample from the cartridge (100) is performed by means of a hydraulic system.

10 15. The method according to any of claims 13 to 14, characterised in that the magnetic force is deactivated prior to the removal of the blood sample.

16. The method, according to any of claims 13 to 15, the method being carried out in a device according to any of claims 1 to 5.

15 17. A system for automatically capturing, detecting, label-free identifying, counting and optionally recovering live circulating tumour cells from blood samples of cancer patients, characterised in that it comprises a cartridge, according to any of claims 7 to 12 and a device, according to any of claims 1 to 6.

20

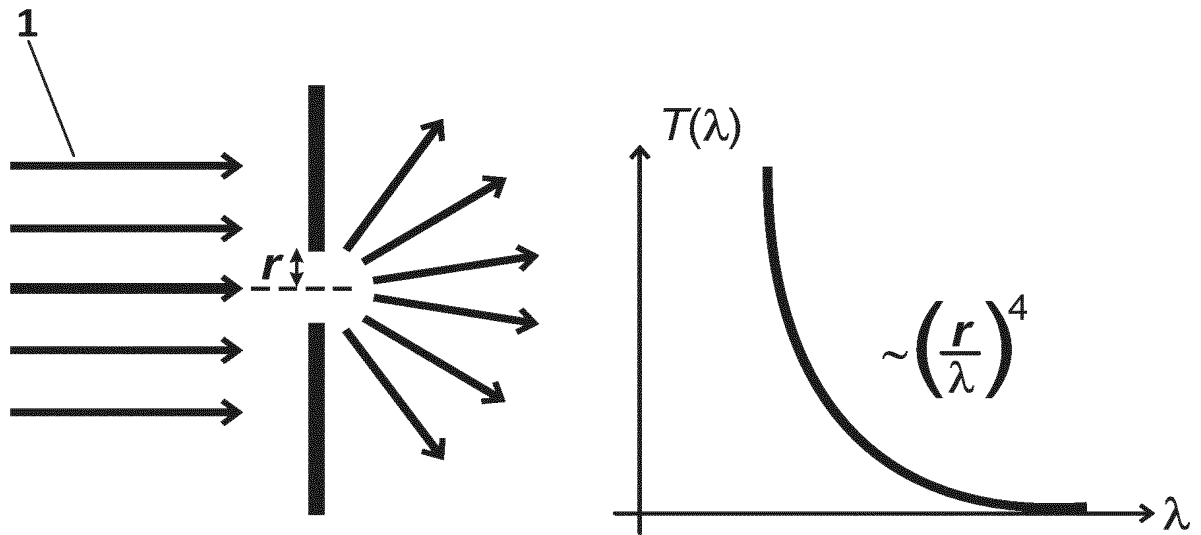


FIG. 1a

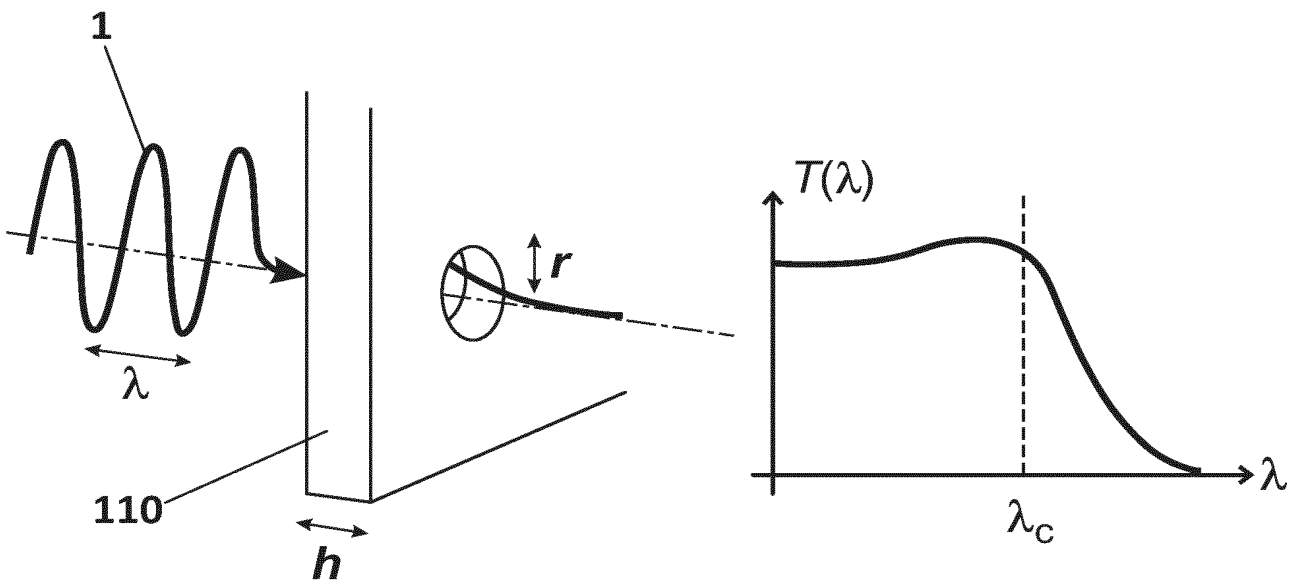


FIG. 1b

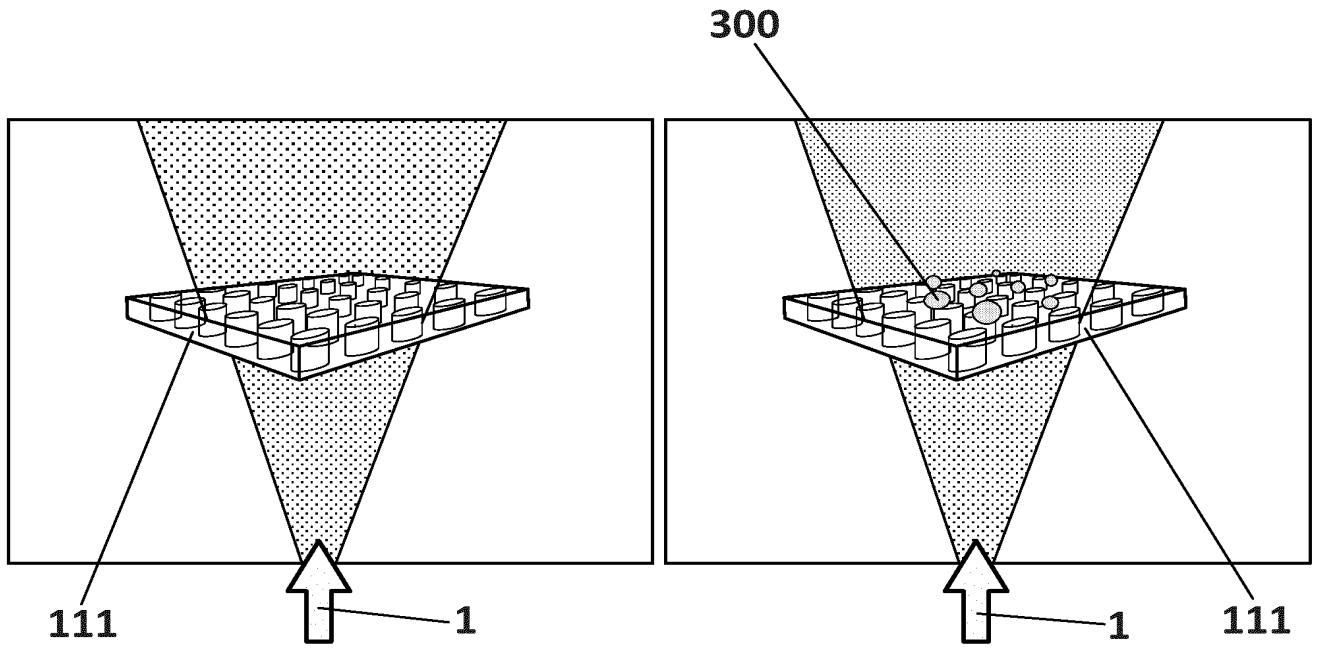


FIG. 2a

FIG. 2b

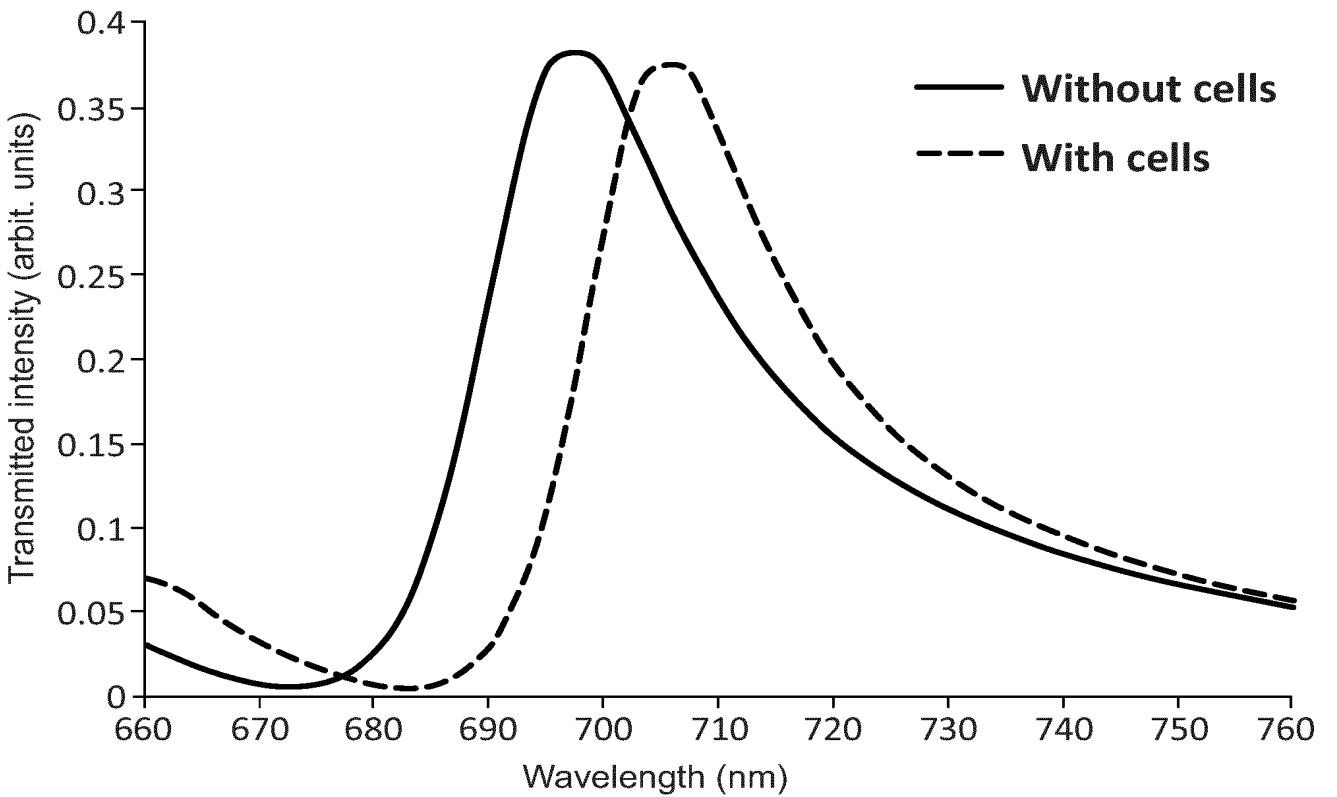


FIG. 2c

3/19

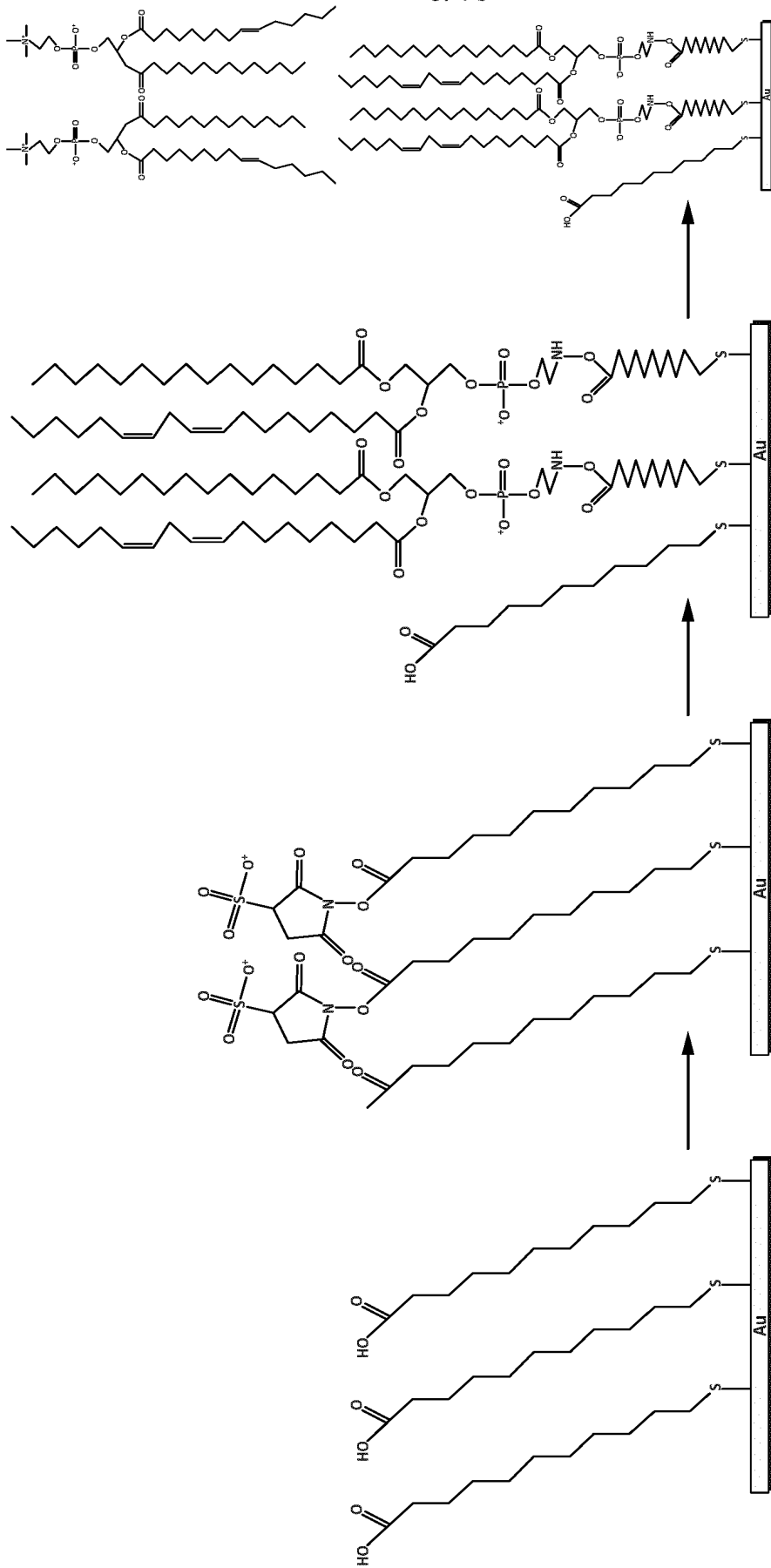


FIG. 3

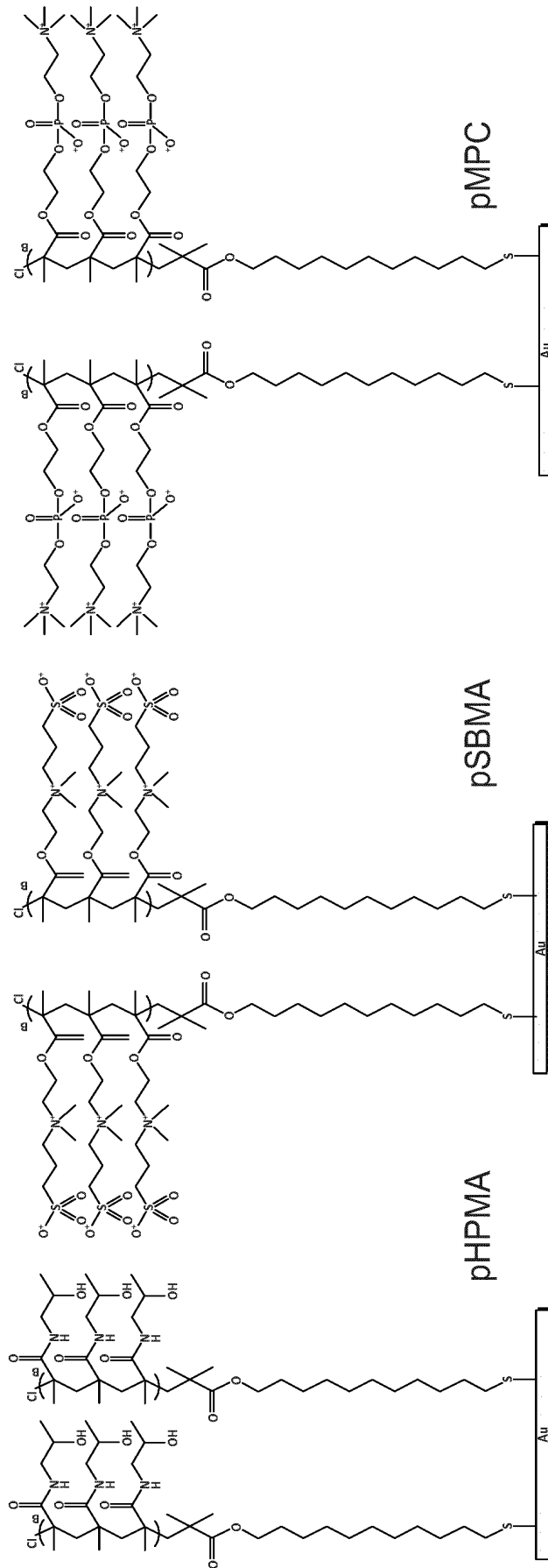


FIG. 4

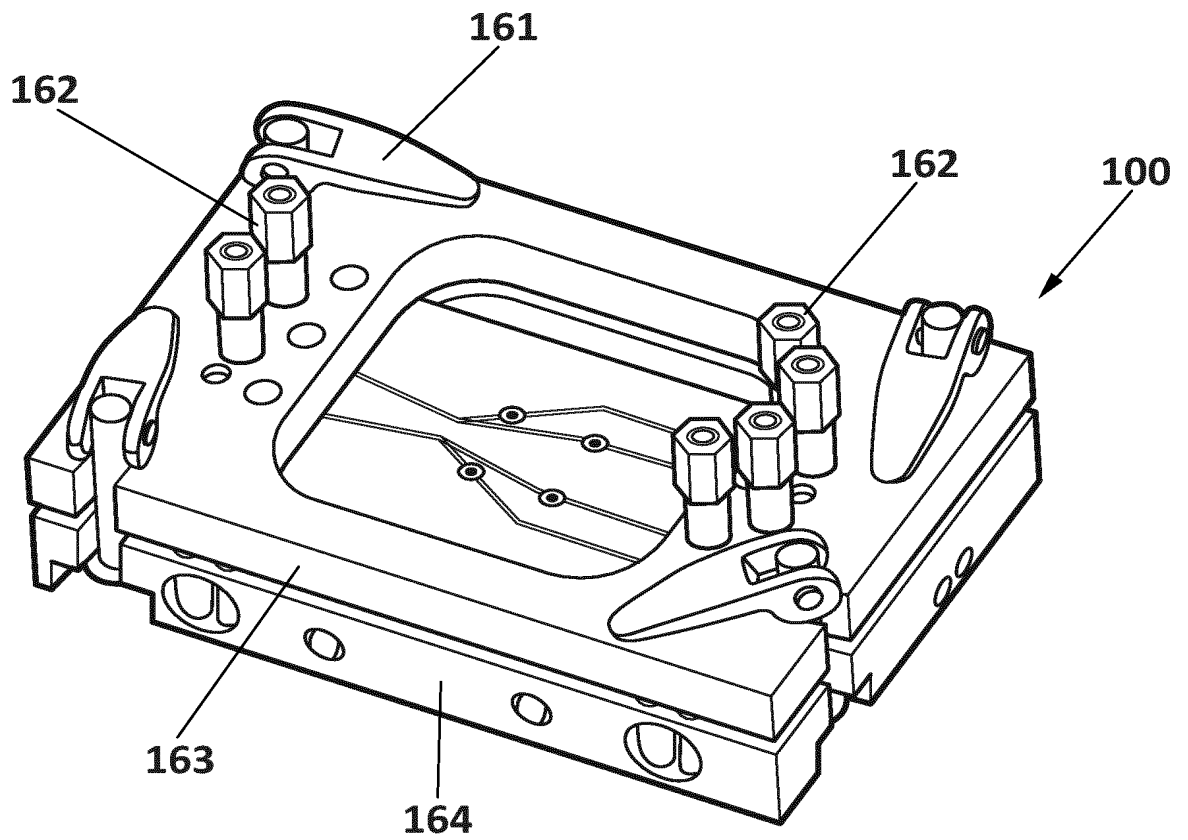


FIG. 5

6/19

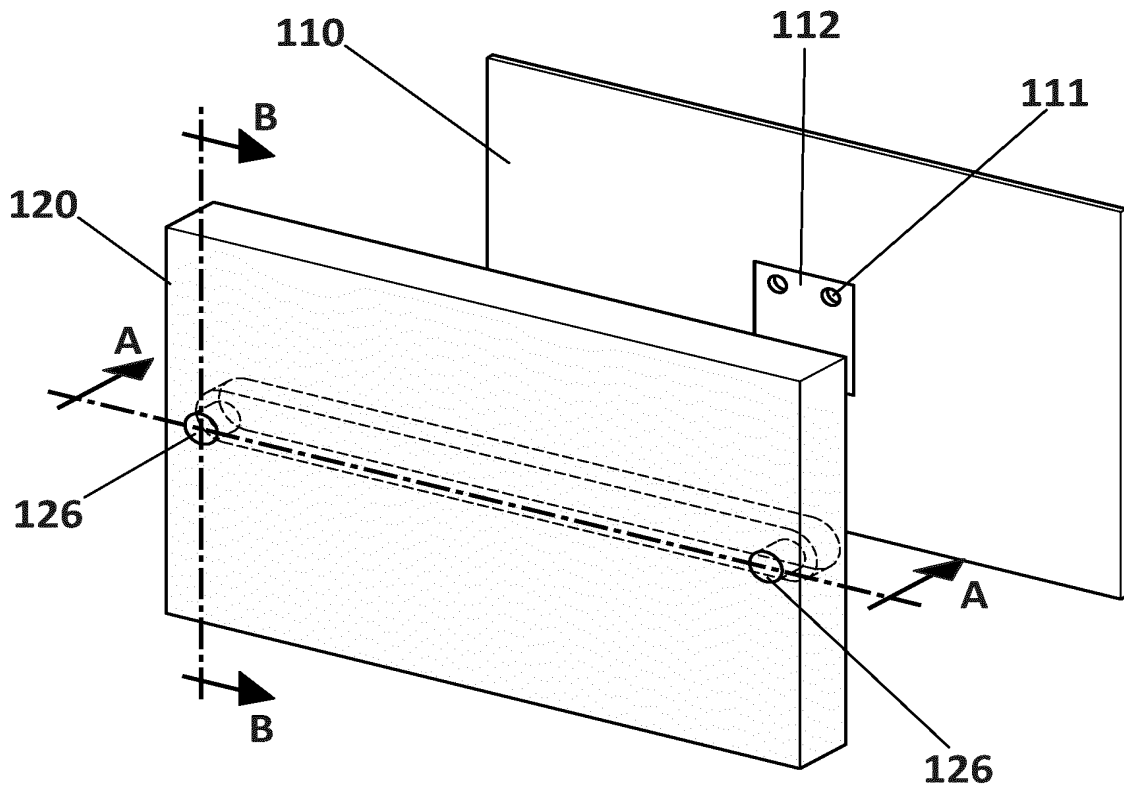


FIG. 6A

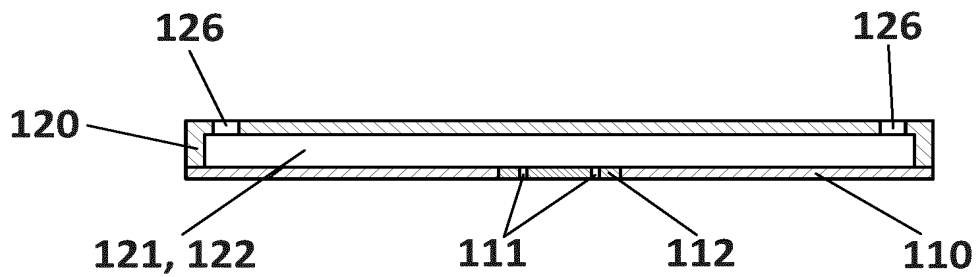


FIG. 6B

A-A

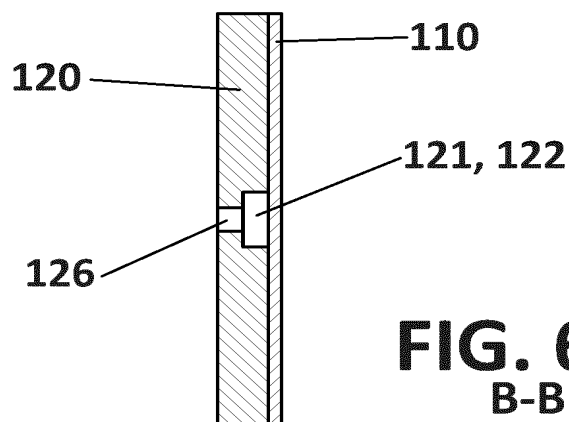


FIG. 6C

B-B

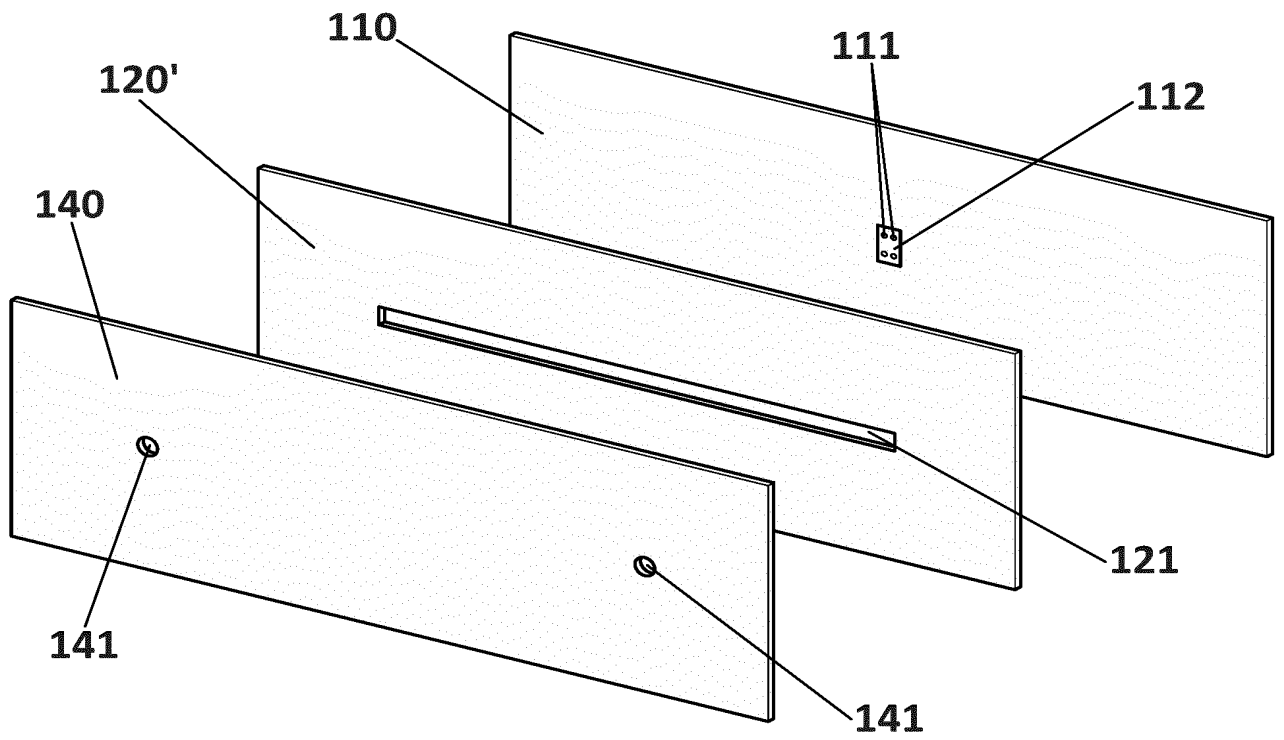


FIG. 6D

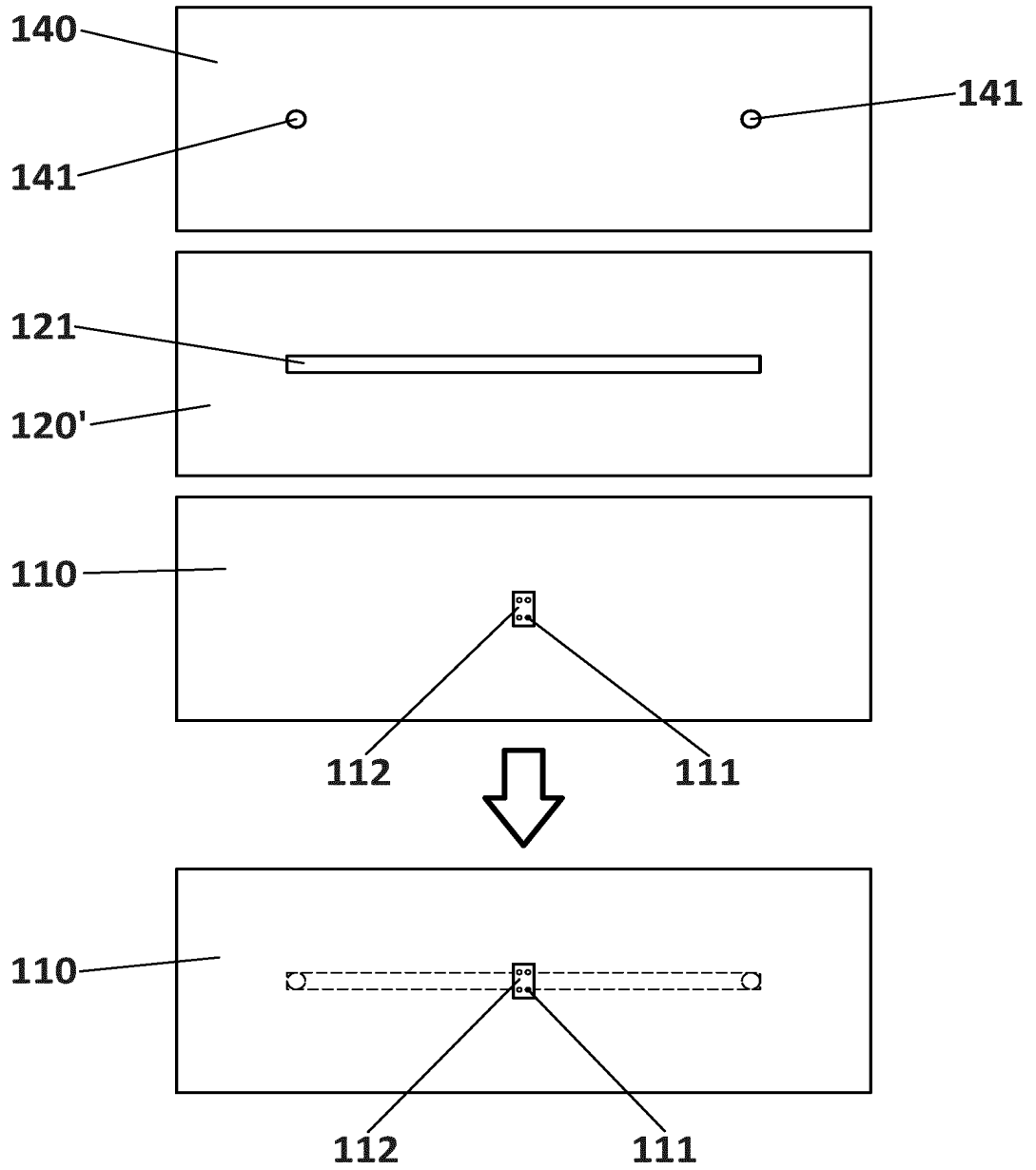


FIG. 6E

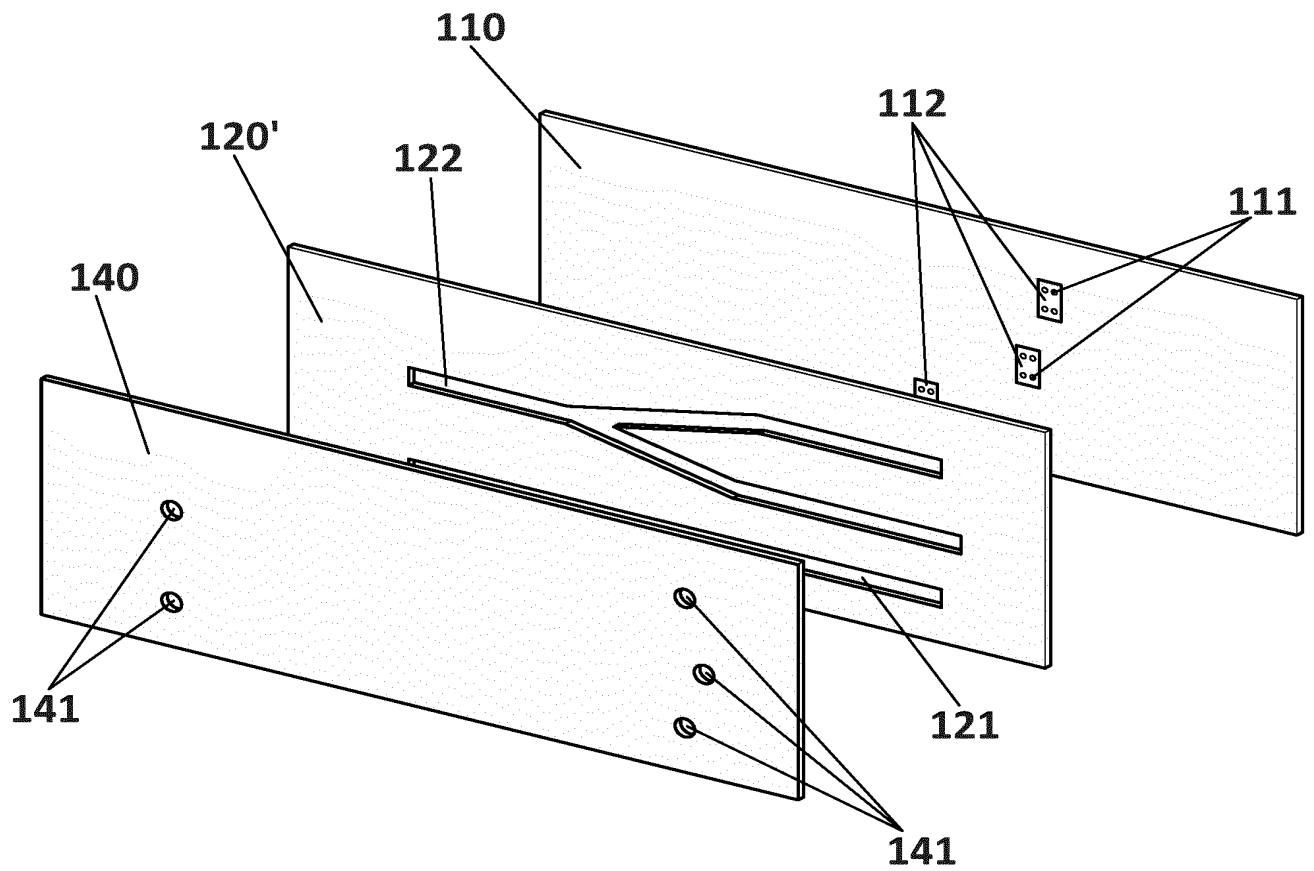


FIG. 6F

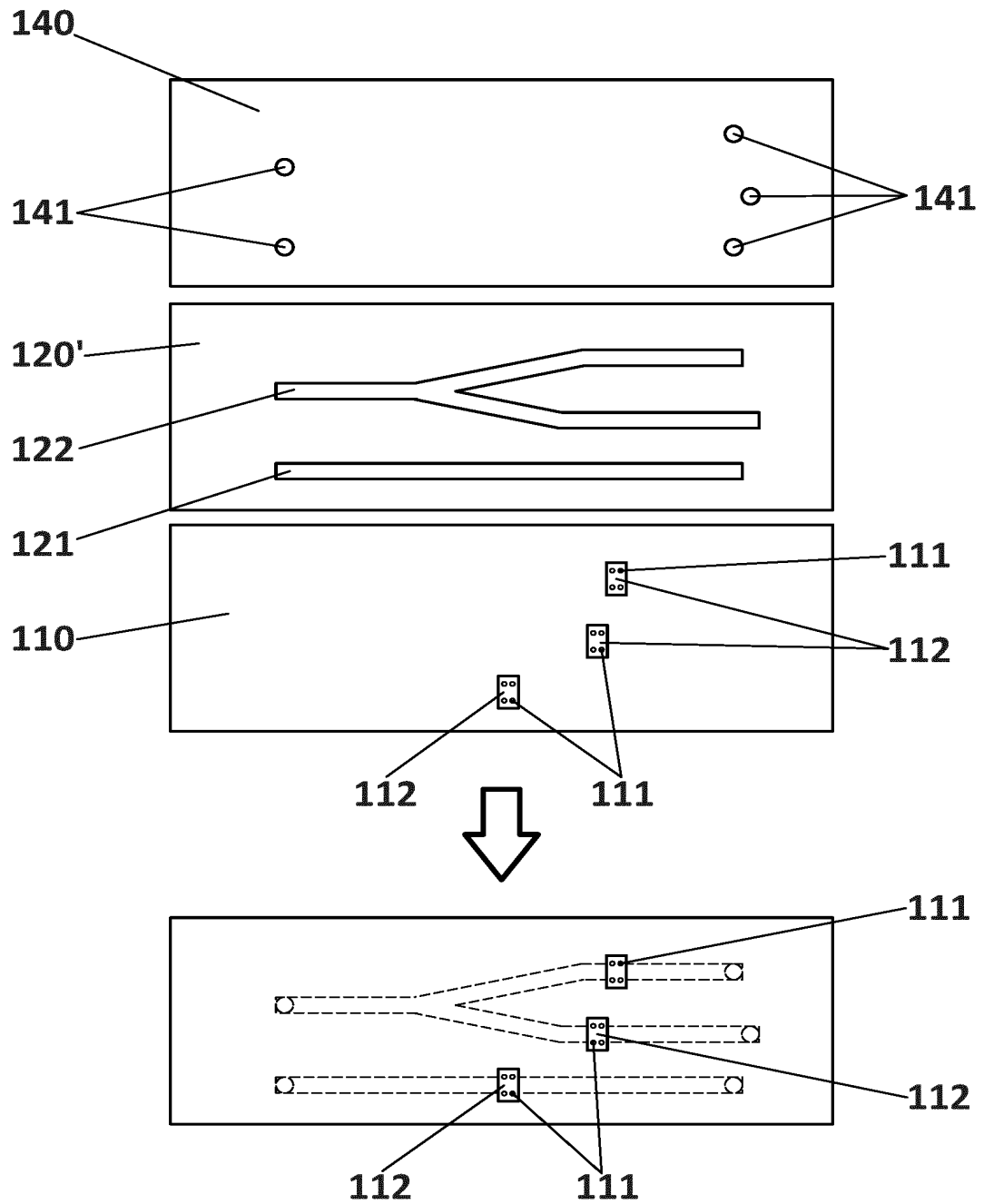


FIG. 6G

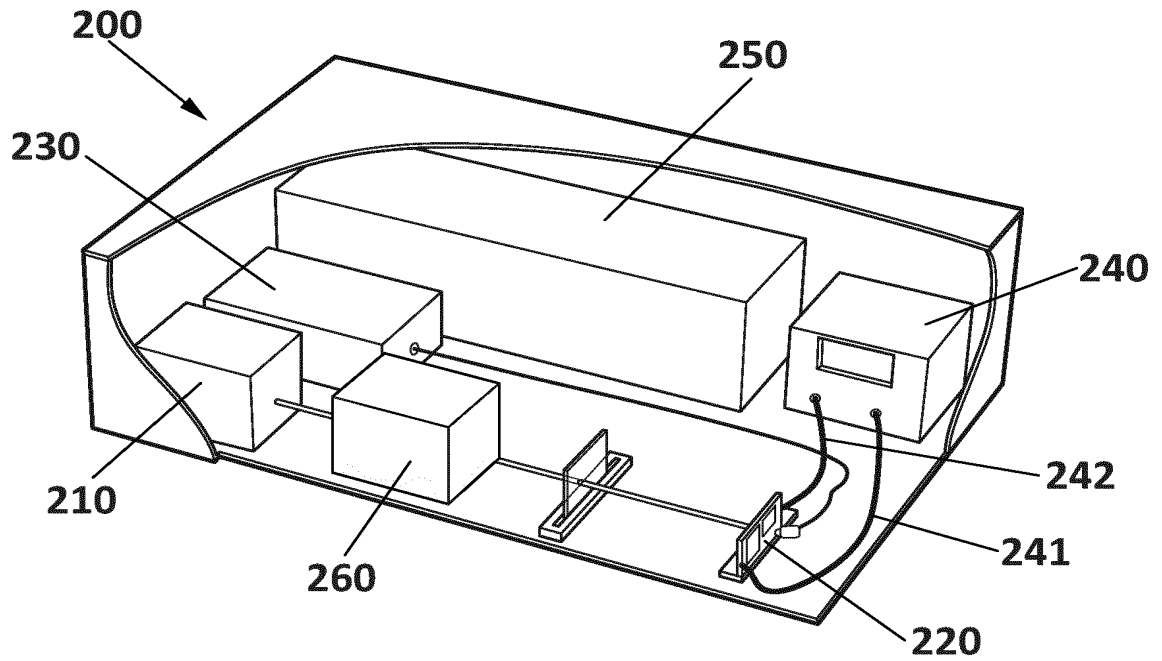


FIG. 7

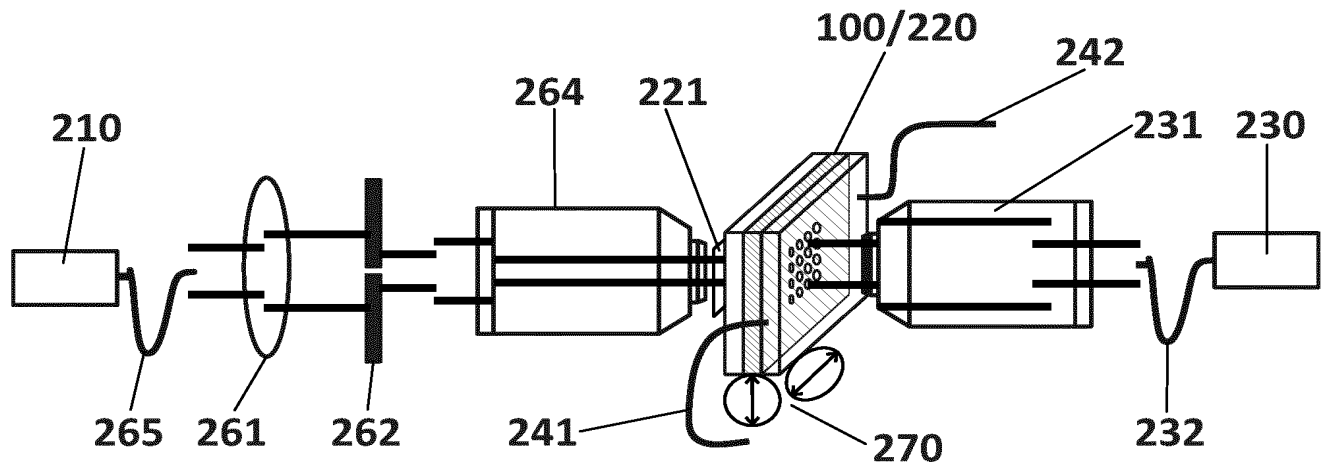


FIG. 8

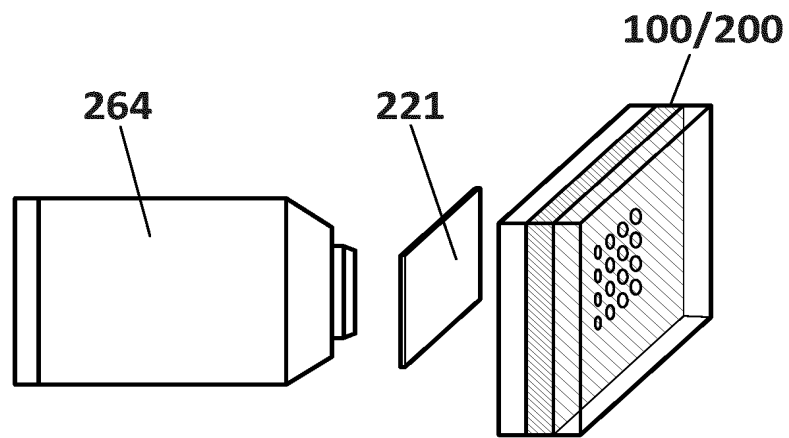


FIG. 9A

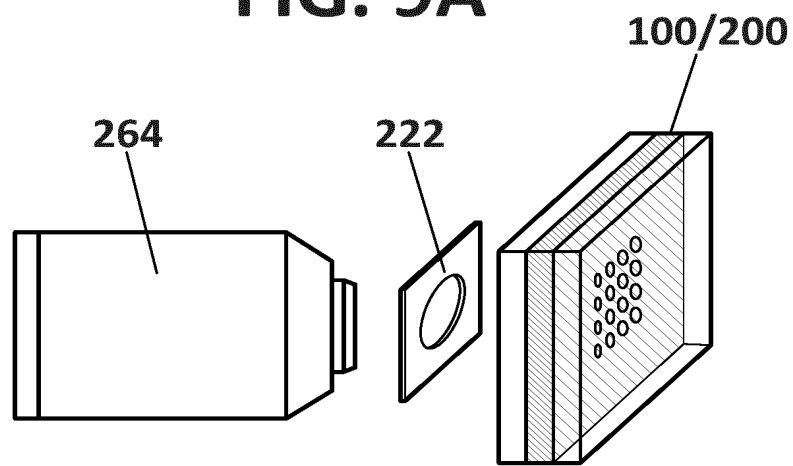


FIG. 9B

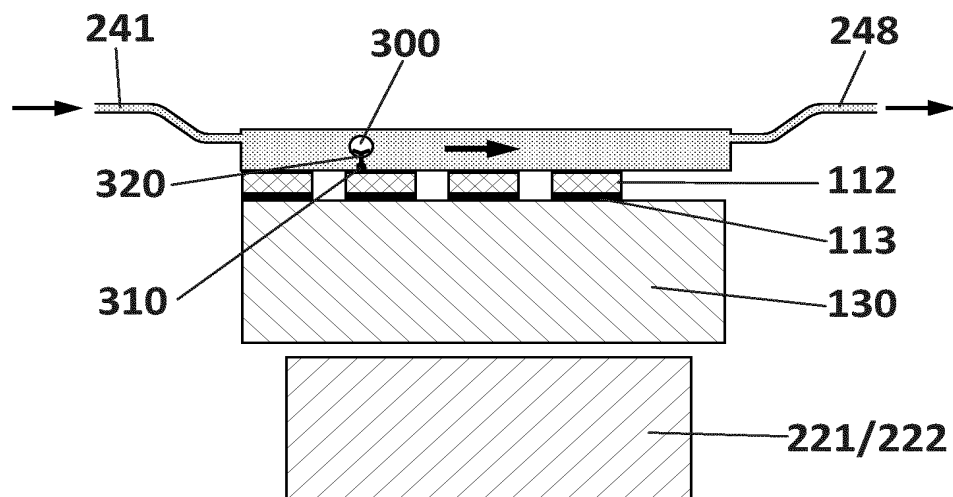


FIG. 10

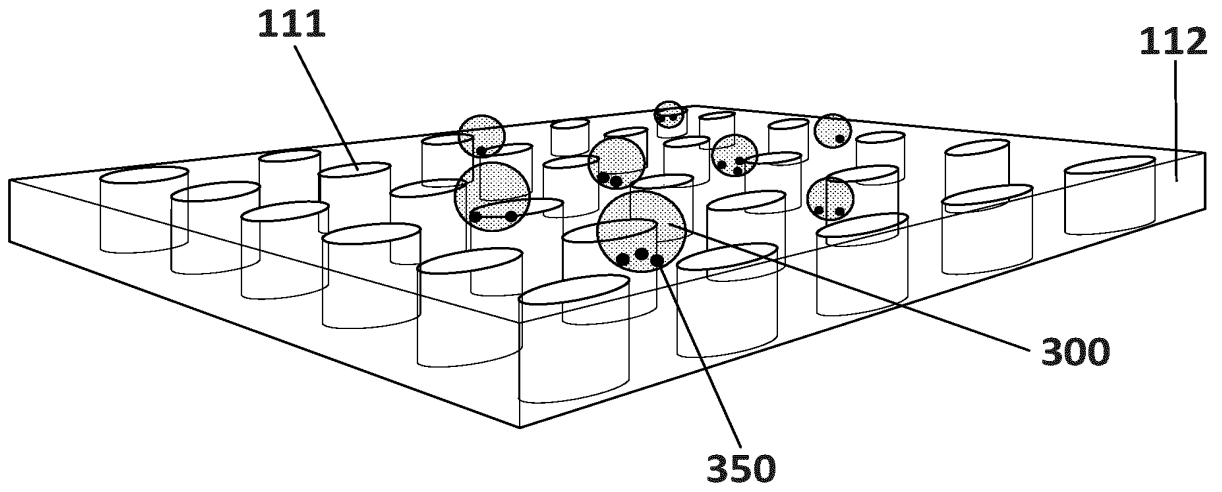


FIG. 11

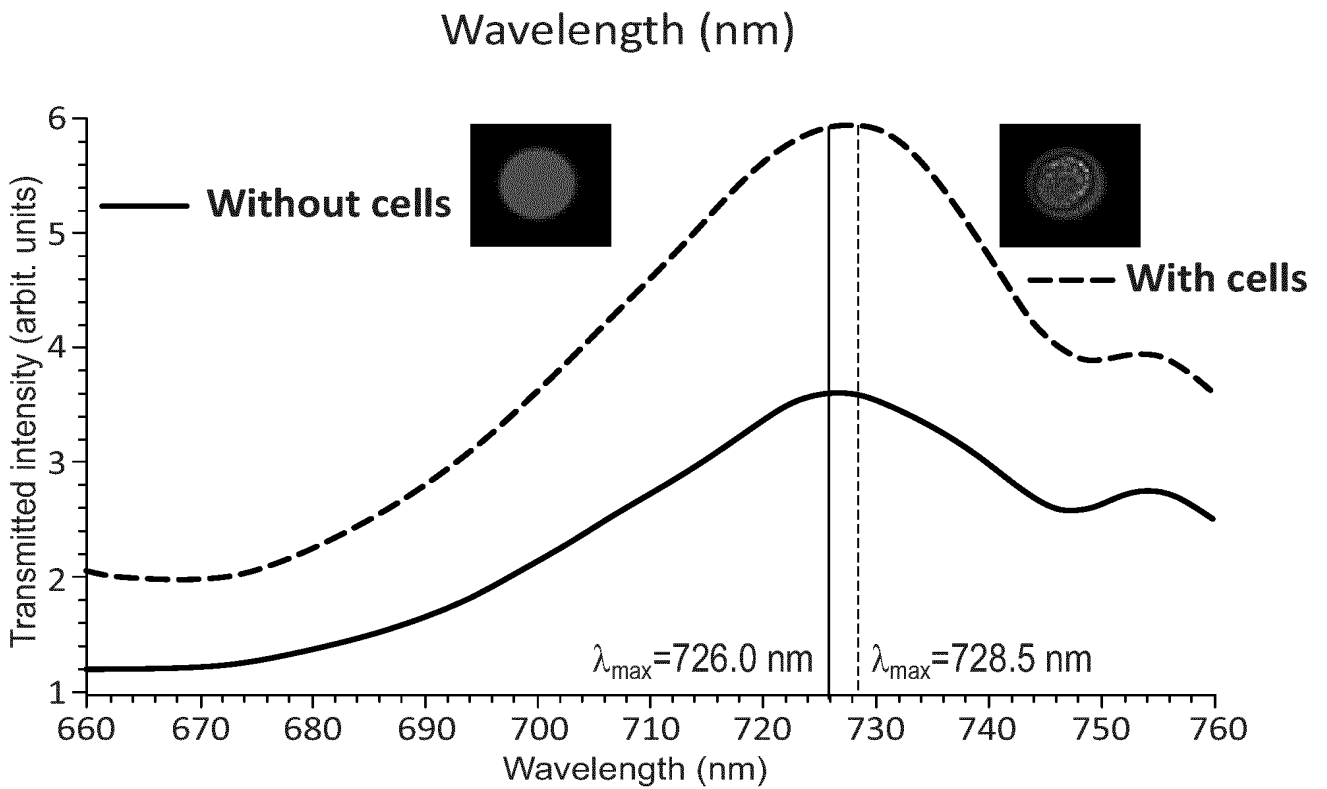


FIG. 12

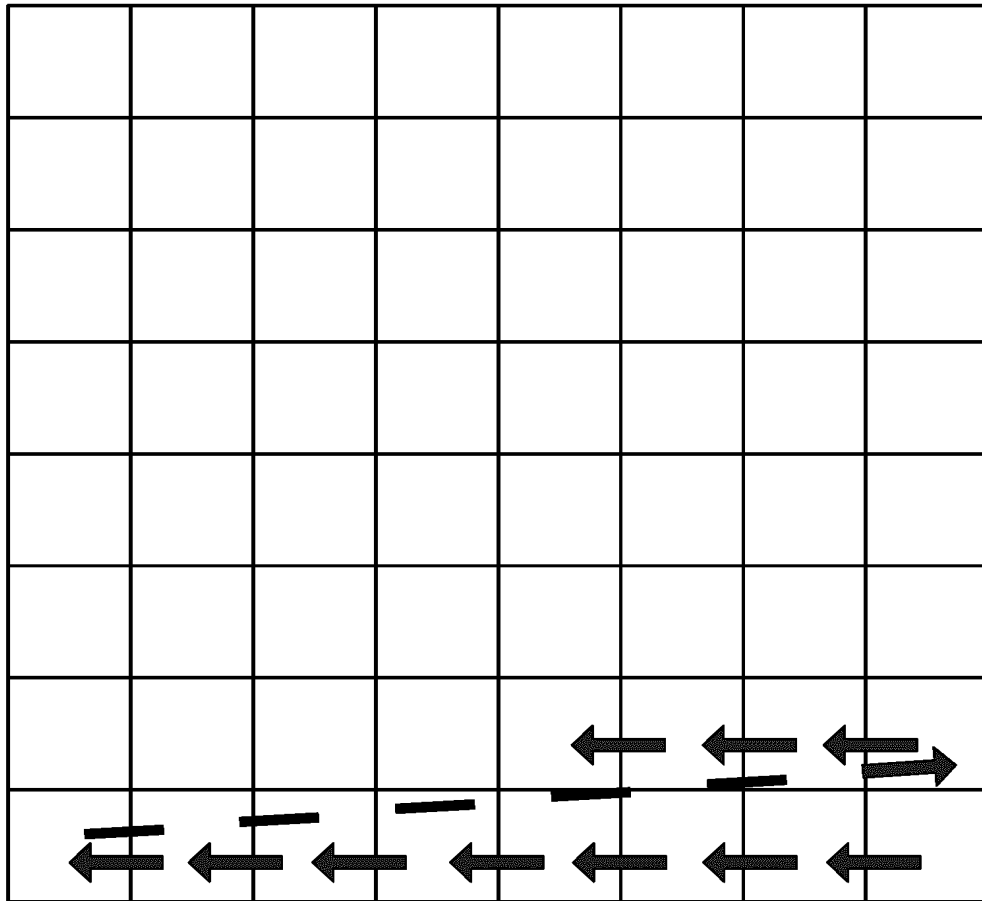


FIG. 13

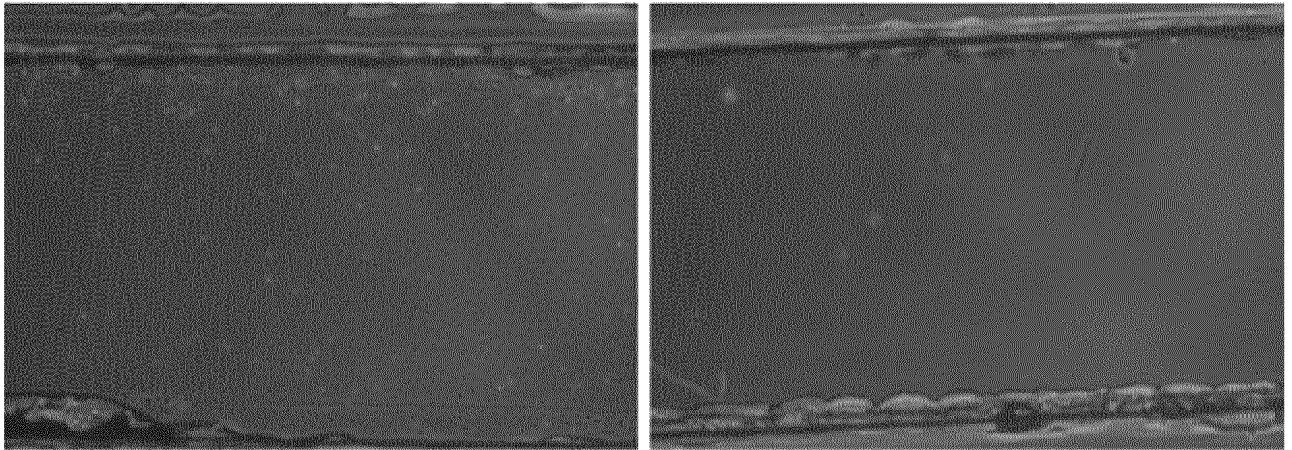


FIG. 14A

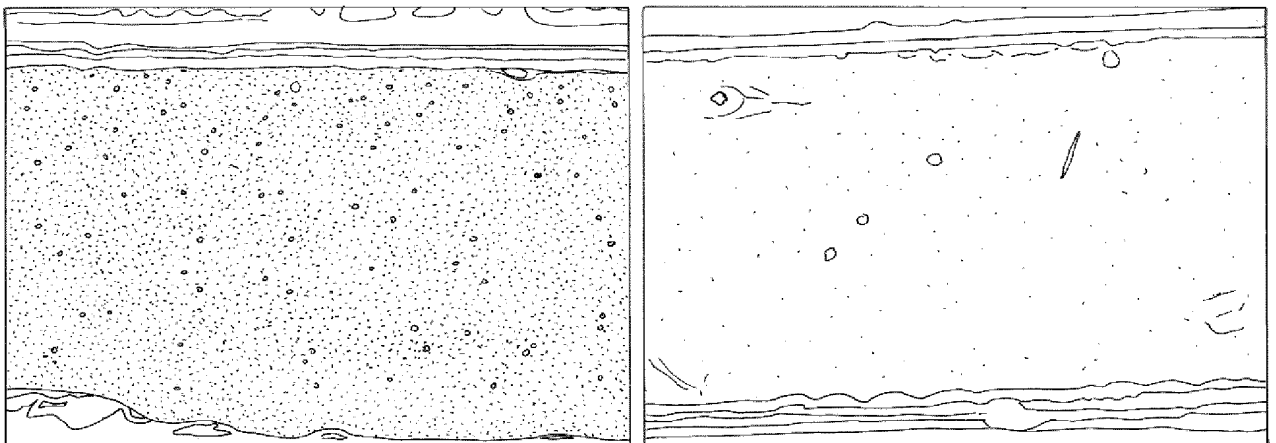


FIG. 14B

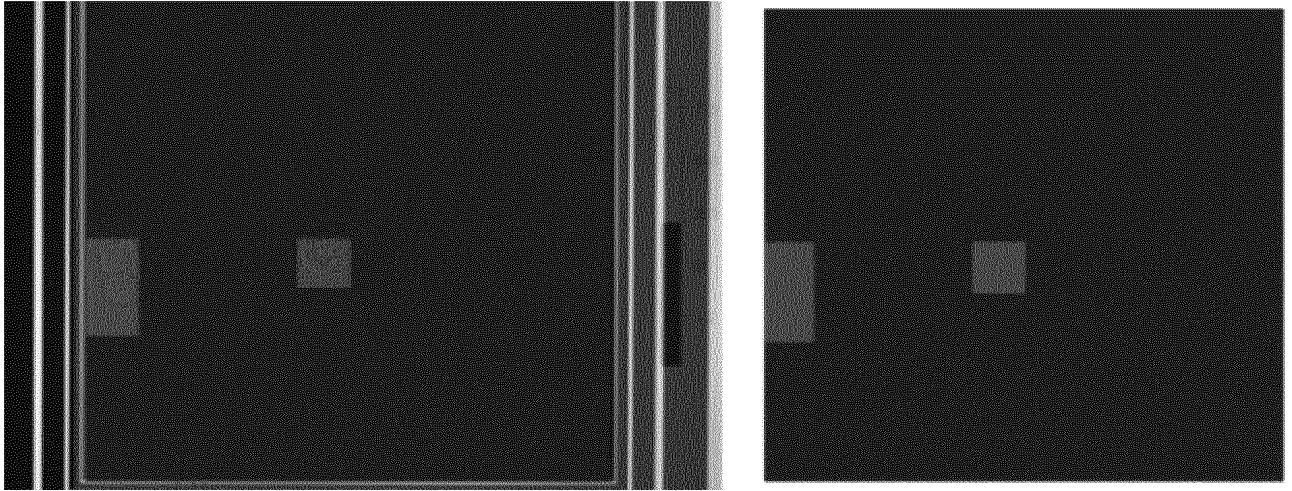


FIG. 15A

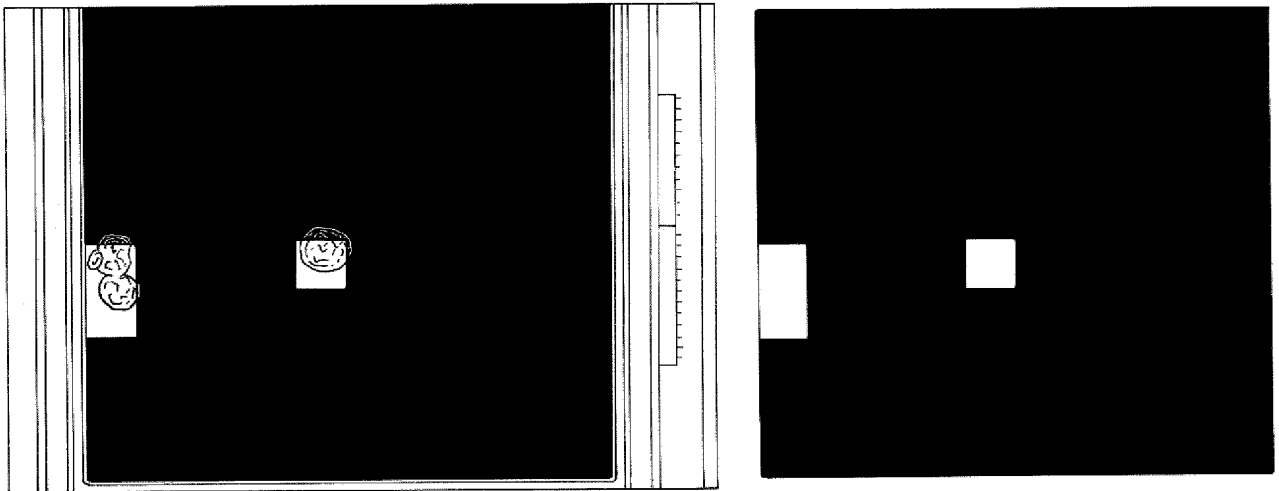


FIG. 15B

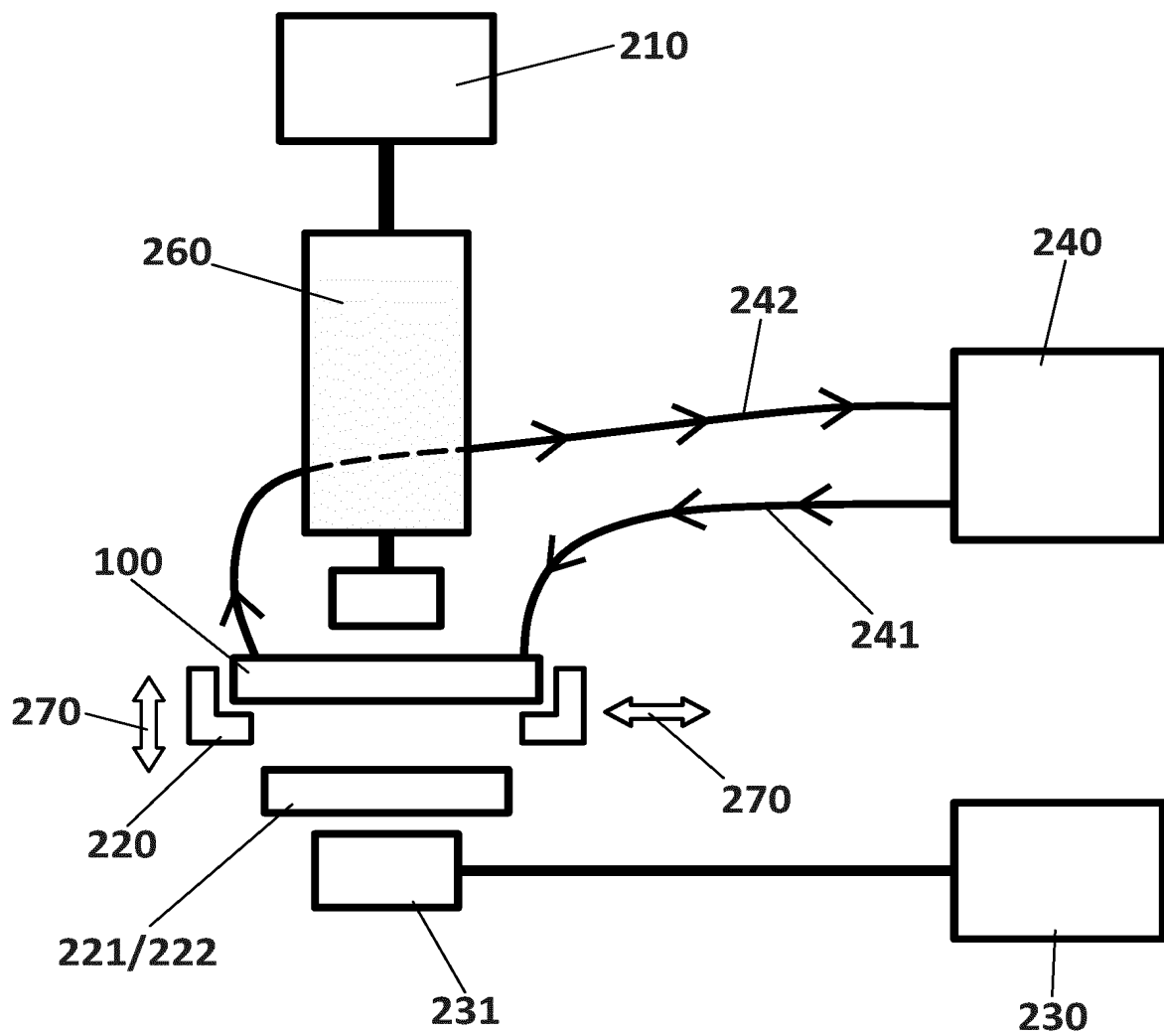


FIG. 16

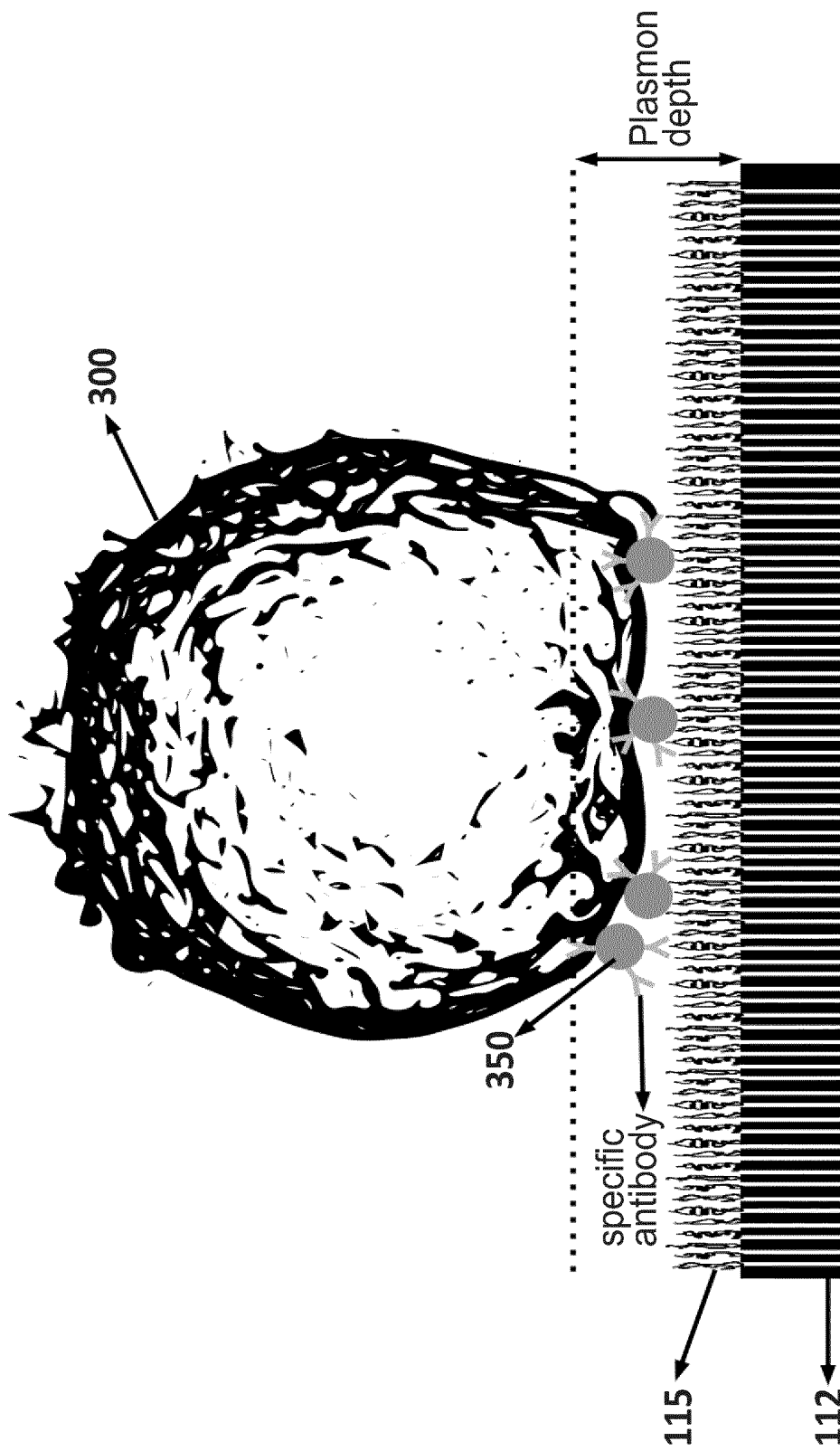


FIG. 17

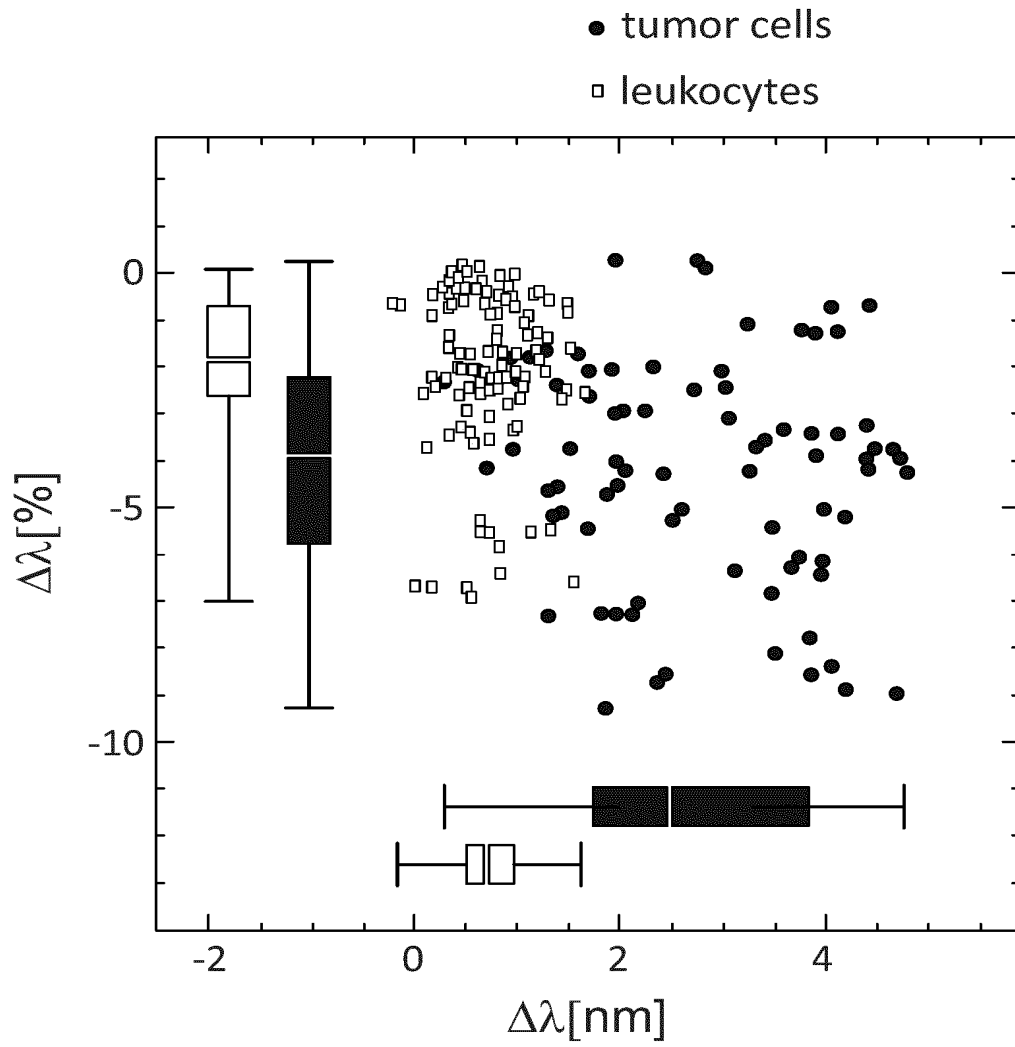


FIG. 18

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/070724

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N35/00 G01N33/543 G01N33/574 B01L3/00 G01N21/552
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 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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2017/199184 A1 (CHANG YING-CHIH [TW] ET AL) 13 July 2017 (2017-07-13) abstract; figures 6A-7H paragraphs [0049] - [0076], [0085] - [0098], [0129] - [0133] -----	1-17
Y	EP 3 121 587 A1 (UNIV CANTABRIA [ES]; SERVICIO CANTABRO DE SALUD [ES]; FUNDACIÓN INST D) 25 January 2017 (2017-01-25) abstract; figures 1A-19 paragraphs [0014], [0036], [0037], [0039], [0045], [0048], [0050], [0054], [0060], [0061], [0066], [0071], [0072], [0092], [0108] ----- -/--	1-17

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

17 October 2018

Date of mailing of the international search report

23/10/2018

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Authorized officer

Zarowna-Dabrowska, A

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/070724

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SARAH UNSER ET AL: "Localized Surface Plasmon Resonance Biosensing: Current Challenges and Approaches", SENSORS, vol. 15, no. 7, 2 July 2015 (2015-07-02), pages 15684-15716, XP055300337, DOI: 10.3390/s150715684 abstract page 15692 - page 15693 page 15703 - page 15704 -----	1-17
Y	MANSOUREH MOUSAVI ET AL: "Label-Free Detection of Rare Cell in Human Blood Using Gold Nano Slit Surface Plasmon Resonance", BIOSENSORS, vol. 5, no. 1, 23 March 2015 (2015-03-23), pages 98-117, XP055443214, CH ISSN: 2079-6374, DOI: 10.3390/bios5010098 abstract; figures 1, 2, 5-7, A2 page 100 - page 111 -----	1-17
Y	MANSOUREH Z. MOUSAVI ET AL: "Magnetic nanoparticle-enhanced SPR on gold nanoslits for ultra-sensitive, label-free detection of nucleic acid biomarkers", ANALYST, vol. 138, no. 9, 28 February 2013 (2013-02-28), page 2740, XP055335696, ISSN: 0003-2654, DOI: 10.1039/c3an36655c abstract; figures 2a, 2b page 2742 - page 2743 -----	1-17
A	EP 2 653 903 A1 (FOM INST FOR ATOMIC AND MOLECULAR PHYSICS [NL]) 23 October 2013 (2013-10-23) abstract; figure 6 paragraph [0060] -----	1-17
Y	HANA VAISOCHEROVÁ ET AL: "Ultralow Fouling and Functionalizable Surface Chemistry Based on a Zwitterionic Polymer Enabling Sensitive and Specific Protein Detection in Undiluted Blood Plasma", ANALYTICAL CHEMISTRY, vol. 80, no. 20, 15 October 2008 (2008-10-15), pages 7894-7901, XP055083280, ISSN: 0003-2700, DOI: 10.1021/ac8015888 abstract page 7895 - page 7896 -----	1-17
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/070724

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2014/315760 A1 (RATNER DANIEL M [US] ET AL) 23 October 2014 (2014-10-23) abstract paragraphs [0037], [0041], [0042], [0054] - [0061], [0075], [0088], [0089], [0092], [0094], [0131] - [0149] -----	1-17
Y	US 2009/181857 A1 (WEI PEI-KUEN [TW] ET AL) 16 July 2009 (2009-07-16) abstract; figure 6 paragraphs [0036] - [0038] -----	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2018/070724

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