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(54) Title: TWIN-FOCUS PHOTOTHERMAL CORRELATION SPECTROSCOPY METHOD AND DEVICE FOR THE CHARACTERIZATION OF DYNAMICAL PROCESSES IN LIQUIDS AND BIOMATERIALS WITH THE HELP OF ABSORBING MARKERS

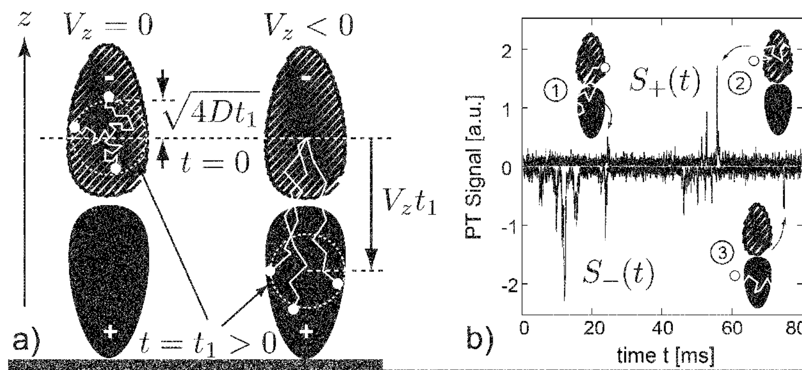

Figure 3

(57) Abstract: The invention relates to a method and a device for twin-focus photothermal correlation spectroscopy for the characterization of dynamical processes in liquids and biomaterials with the help of absorbing markers. Thereby non-fluorescent absorbing nano objects are heated by an intensity-modulated heating laser which leads to a refractive index gradient lens around the object. This refractive index gradient is detected by a detection laser with a focal volume that, depending on the position of the heated object relative to the focal plane of the detection beam, splits into two-sub-volumes forming a twin-focus comprising two sharply separated parts of a focal volume showing no spatial overlap.

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Twin-focus photothermal correlation spectroscopy method and device for the characterization of dynamical processes in liquids and biomaterials with the help of absorbing markers

The method according to the invention allows the measurement of dynamical processes in liquids, liquid mixtures and complex soft materials such as biological materials or living cells with the help of a split focal volume (twin focus) in photothermal detection, in particular in photothermal correlation spectroscopy. The well separated focal volumes, precisely sub-volumes show no spatial overlap and thus allow the study of isotropic as well as anisotropic processes in biophysics, biology, physics, chemistry and medicine (e.g. high throughput screening).

Recent developments in the field of optical microscopy and detector technology have given access to the fluorescence of single molecules allowing to study dynamical processes on the nanoscale. This has become especially important in the field of biophysics/biochemistry where single fluorescent molecules are used as markers/tracers to follow biologically relevant processes. The most promising applications involve the detection of dynamical processes in living cells such as the infection pathways a virus takes entering the cell and cell nucleus [1], as single molecule techniques provide local information due to the small size of the markers (nanometer scale). During the approximately 20 years of single molecule fluorescence detection a large number of different experimental techniques have been developed. An especially useful variant of fluorescence detection is fluorescence correlation spectroscopy (FCS) [2]. Commonly, FCS is employed in the context of confocal or multi-photon excitation microscopy. In these techniques light is focused on a sample and the measured fluorescence intensity fluctuations (due to diffusion, physical or chemical reactions, aggregation, etc.) are recorded and analyzed using the temporal autocorrelation. Because the measured property is essentially related to the magnitude and/or the amount of fluctuations, there is an optimum measurement regime at the level when individual species enter or exit the observation volume (or turn on and off in the volume). When too many entities are measured at the same time the overall fluctuations are small in comparison to the total signal and may not be resolvable – in the other direction, if the individual fluctuation-events are too sparse in time, one measurement may take prohibitively too long. FCS is in a way the fluorescent counterpart to dynamic light scattering, which uses coherent light scattering, instead of (incoherent) fluorescence.

When an appropriate model for the analysis of the temporal autocorrelation is known, FCS can be used to obtain quantitative information such as

- diffusion coefficients
- hydrodynamic radii
- average concentrations
- kinetic chemical reaction rates
- singlet-triplet dynamics
- molecular binding kinetics
- molecular binding constants.

As fluorescent markers come in a variety of colors and can be specifically bound to a particular molecule (e.g. proteins, polymers, metal-complexes, etc.), it is possible to study the behavior of individual molecules. With the development of sensitive detectors such as avalanche photodiodes the detection of the fluorescence signal coming from individual molecules in highly dilute samples has become practical. With this emerged the possibility to conduct FCS experiments in a wide variety of specimens, ranging from materials science to biology. The advent of engineered cells with genetically tagged proteins (like green fluorescent protein) has made FCS a common tool for studying molecular dynamics in living cells.

Despite these successful applications, FCS suffers from a number of drawbacks, which are either due to the use of organic chromophores itself or due to technical limitations set by the optical detection and excitation scheme used in confocal microscopy.

The drawbacks of organic chromophores, fluorescent probes or semiconductor quantum dots are related to the following facts

- dye molecules have to be specifically designed especially in the case when they shall be bound to biological structures
- dye molecules need to have a large emission quantum yield, which however depends on the local environment
- dye molecules shall not undergo chemical reactions with molecular species involved in the processes under investigation
- dye molecules as fluorescent markers/tracer shall not interfere with the function of molecular species they tag

- dye molecules undergo photochemical processes which lead to an irreversible bleaching
- dye molecules obey a complex intermittent emission behavior known as blinking, which prevents the emission of photons during time period between microseconds and seconds
- dye molecules have a limited photon emission rate.

These drawbacks of organic fluorescent marker molecules have been resolved by employing absorbing particles instead of organic dye molecules. Absorbing particles do not suffer from most of the photophysical limitations described above. However, as they do not emit fluorescence photons they cannot be detected in fluorescence microscopy. Thus other techniques for the detection of single absorbing particles have been developed. Among the direct detection by light scattering, small particles below a size of a few 10 nm are not visible in light scattering microscopy especially in inhomogeneous environments such as living cells. A relatively new detection method for single absorbing nanoparticles is photothermal microscopy [3]. Photothermal detection techniques employ an optical heating of particles in a confocal microscope and the release of heat from these particles for their detection. The released heat induces a local refractive index change, which is monitored by a second focused laser, which is not absorbed by the particle. Such photothermal detection methods have, for example, already been used to track single gold particles in solution [4].

Above that, the WO 2004/025278 A1 describes a photothermal detection method based on a dual laser focus and differential interference contrast. In this method two laterally displaced focal volumes are used to probe the dynamics of a particle for random and oriented motion. Thereby, the two focused laser beams are spatially separated before they pass through the sample. In this way, both lasers experience a different phase shift as they interact, or not, with the sample, precisely with the thermally induced refractive index variation. Subsequently, the two laser beams are brought to interfere on an appropriate detector, whereby the detected signal intensity depends on the phase difference between the lasers. Because of the lateral displacement of the laser foci and the need to separate the detection lasers in probe and reference beam, this method is not easy to integrate in a microscopic setup. Furthermore, the diffraction-limited laser beams will always overlap which leads to an uncertainty when it comes to the detection of directed motion. Spatial information on the position of a probe in one of the focal volumes itself can also not be obtained with this method.

As the heating of small particles can be carried out very quickly (MHz), lock-in techniques can be used to differentiate between scattering and absorbing particles in the sample. These techniques have been demonstrated in a correlation spectroscopy mode (largely equivalent to FCS) as well and are termed photothermal correlation spectroscopy (PhoCS) [5].

Such photothermal correlation spectroscopy has, for example, already been used to analyze the free diffusion of gold nanoparticles in solution. Therefore, the correlations of an interferometric photothermal signal have been analyzed. In particular, the autocorrelation function of the time traces of the photothermal signal was determined and information about the diffusion dynamics of the absorbing particles across the focus has been obtained [6]. Furthermore, such method has been used for the precise determination of the hydrodynamic sizes of different functionalized gold nanoparticles [7]. Thus, in analogy to standard fluorescence correlation spectroscopy (FCS) the autocorrelation function cannot provide information about directed motion of the tested particles.

By using absorbing nanoparticles instead of organic dye molecules as described above, most of the chromophore related drawbacks of FCS have been removed. Still, technical drawbacks of FCS related to the confocal microscopy setup itself remain, especially

- the confocal detection volume is limited by diffraction and aberrations
- the confocal detection volume is commonly modeled as an 3-dimensional gaussian volume
- no spatial information on the position of the dye molecule in the focal volume is obtained
- FCS delivers no information on anisotropic dynamics in the focal volume.

The latter drawbacks have been partly removed by techniques modifying the focal volume of fluorescence correlation spectroscopy. For instance, the capability to detect anisotropic or directed motion, which is extremely important for the detection of driven processes in living cells, is introduced in dual focus FCS techniques [5]. Here two laterally displaced detection volumes are employed simultaneously to identify the motion from one focal volume to the other. The used focal volumes do, however, overlap spatially and no well defined border between the focal volumes is possible. Thus, such FCS techniques are not able to discriminate sharply between the two focal volumes, which is especially important for the detection of small velocity directed motions or small amplitude fluctuations (i.e. the motion of

a few 10 nm). This is one of the major drawbacks which are removed by the presented method of twin-focus photothermal correlation spectroscopy.

The detailed drawbacks of current methods are based on

- the use of organic fluorescent probes
- the detection of fluorescence photons.

The objective of the invention is to overcome these drawbacks. This objective is solved by the method according to claim 1 and a device according to claim 10. Claim 2 to 9 and 11 to 13 refer to preferred embodiments of the invention.

Within the twin focus photothermal correlation spectroscopy approach according to the invention, the following drawbacks of fluorescence correlation spectroscopy are removed:

- the need for specifically designed fluorescent molecules
- the lack of long term photostability of the markers under investigation
- the contribution of complex intermittent emission of the markers to the correlation signal
- the lack of spatial information on the particle position
- spatial overlap of detection volumes in multi-focus FCS approach.

The following sources of these drawbacks are eliminated by the invention

- the use of fluorescent probes
- the use of fluorescence microscopy
- the specific generation of multiple focal volumes for the detection of anisotropic motion.

The method according to the invention is based on single molecule photothermal microscopy methods as developed by Lounis and others [9 - 11]. It preferably employs a photothermal microscopy setup in transmission configuration as shown in Figure 1. The device according to the invention comprises two lasers sources, which are used as the light sources for heating the absorbing nano objects (in the following called heating laser or excitation laser), e.g. nano particles or molecules, and detecting a change in the refractive index around the

heat releasing nano object (in the following called detection laser or probe laser). The heating laser is intensity modulated at a frequency between 100 kHz and 1 MHz and preferable up to 10 MHz. The detection laser is used in continuous wave (cw) mode. Both lasers paths are merged and focused by an objective lens to the sample. To generate a twin focus volume (see Figure) both heating and detection laser focal volume have to overlap, in a preferred embodiment both heating and detection laser focal plane have to overlap at the same focal point.

An absorbing nano object, e.g. a particle, entering this focal volume of the heating laser will cause a divergent gradient index thermal lens around the object, resp. the particle. Depending on the position of the nano object, e.g. the particle, with respect to the focus of the detection laser, the detection beam will become either wider or narrower. The collected detection laser light, which is imaged by a detection lens, e.g. a second microscopy objective lens, on a photo detector, e.g. a photodiode, is thus either decreased or increased.

As the heating laser is modulated in intensity the detection laser scattering signal, and so the intensity at the photodetector, will become modulated as well, however, only if a nano object, e.g. a particle, including the thermal lens is in the focus of the detection laser. Thus the signal of the photo detector, e.g. a photodiode, can be analyzed at the heating laser intensity modulation frequency, for example by a lock-in amplifier, to remove unmodulated contributions when no nano object, e.g. no particle, is in the focus.

In this example, the lock-in amplifier will provide the amplitude and information about the relative phase of the detection intensity modulation to the heating laser intensity modulation. The phase information will provide the required information on the position of the nano object, e.g. a particle, in one of the two parts of the focal volume. While the photothermal microscopy technique has been known and applied so far, the lens like action and the splitting of the focal volume into two sub-volumes (twin focus) has not been known on a single particle level so far.

In the context of the twin focus photothermal correlation spectroscopy approach described here, the twin focus volume refers to the focal volume of the detection laser that splits into two sub-volumes in the presence of a heated nano object. Thereby, the twin focus differs from the already known dual focus methods, as the twin focal volumes, precisely the sub-volumes, show no spatial overlap but also no spatial gap between each other. On the contrary, they are adjacent to each other and together they form the focal volume of the detection laser.

As the heated nano object acts as a divergent gradient index thermal lens, the beam of the detection laser is configured depending on the relative position of the nano object and the focal plane of the detection beam. When the nano object is in propagation direction of the detection laser in front of its focal plane, the lens-like action of the nano object narrows the beam of the detection laser at the aperture of the ~~collection~~ detection lens. Respectively, for a position of the nano object behind the focal plane of the detection laser in its propagation direction the beam is widened. Thereby, the intensity of the detection laser on the photo detector is increased in the first (positive signal) and decreased in the second (negative signal) case. For a nano object in the focal plane of a non-aberrated detection laser the beam would neither be narrowed nor widened and thereby the signal neither increased nor decreased (see Figure 5). In this way a focal volume that splits into two sub-volumes, the twin focus volumes, is generated. These neighboring volumes show no lateral or axial overlap. Consequently, they are well separated, as the modulation of the detection laser intensity modulation, the photothermal signal, shows a zero crossing when the nano object is in the focal plane of the detection laser.

When using a lock-in amplifier for the analysis of the photothermal signal, the magnitude and phase of the lock-in detected signal comprise the positive and negative signal parts. The signal amplitude and spatial size of the neighboring positive and negative sub-volumes is determined by the relative axial displacement of the heating and the detection laser beam focal planes. For example, in the case of non-aberrated heating and detection laser beams overlapping at the same focal point both negative and positive detection volumes will have the same size and signal magnitude. Adjusting the relative axial focal position of the heating to the detection laser beams allows enhancing either the positive or the negative photothermal signal.

Due to optical aberrations common to any microscope setup, the symmetric two lobe structure is not obtained for perfect heating and detection laser beam overlap. Further the particle position in the focal plane will cause a non-zero signal. The effect of the aberrations on symmetry of the detection volume can be compensated by changing the relative axial displacement of the focal planes of the detection and heating laser beam. In this way, by shifting the focal plane, one can not only counter the effects of aberration, but also cause an enhancement of either the positive or the negative signal. The axial offset of the two focal planes is adjusted by at least one additional lens in front of the objective, with which the divergence of either the heating or detection laser can be controlled.

The split focal volume with its positive and negative signal parts is now allowing the detection of particle fluctuations in either one of the volumes or between the sub-volumes.
Thus the new technical component of our invention is based on the understanding of the signal generation in photothermal microscopy to provide the twin focus in single particle detection. In addition we supply the corresponding methods and models to analyze twin focus photothermal signals to measure dynamical processes in liquids and biological materials.

The invention comprises at least one of the following:

- a method of twin-focus photothermal correlation spectroscopy with a twin-focus volume comprising two separated focal volumes, precisely two sub-volumes, showing no spatial overlap.
- the experimental realization of two sharply separated focal volumes, precisely two sub-volumes, (two separated focal volumes, precisely two sub-volumes, showing no spatial overlap) for photothermal correlation spectroscopy
- the detection of phase sensitive signals from the two sharply separated focal volumes, precisely two sub-volumes, in photothermal correlation spectroscopy
- the experimental realization and application of two sharply separated parts, precisely sub-volumes, of a photothermal focal volume to detect anisotropic fluctuations in the concentration of absorbing non-fluorescent markers
- the experimental realization and application of two sharply separated parts, precisely sub-volumes, of a photothermal focal volume to detect directed dynamics of absorbing non-fluorescent markers along the optical axis of a photothermal microscope
- the experimental realization and application of two sharply separated parts, precisely sub-volumes, of a photothermal focal volume to detect spatial differences in the fluctuation of absorbing non-fluorescent markers in liquid and biological samples
- the analysis of the above mentioned processes by temporal correlation functions, which involve the autocorrelation and cross correlation of the signals from delivered by a lock-in amplifier

Advantages of the invention include at least one of the following:

- intrinsic availability of two neighboring and sharply separated measurement volumes
- use of photostable marker/tracer nano objects e.g. of gold or other nobel metal nanoparticles
- measurement at medium light intensities with no requirement for photon counting detectors

The non-fluorescent absorbing particles used as markers in the invention are preferably metal or metaloxid or carbon nanoparticles with a preferred diameter of 1 to 100 nm. The absorbing particles are preferably selected from gold or other noble metal nanoparticles, quantum dots or carbon nanotubes. The use of non-fluorescent absorbing molecules is possible as well.

Absorbing nano objects, e.g. particles or absorbing molecules (or light absorbing particles or molecules), mean in the context of the present invention that the nano objects absorb light and convert the absorbed energy at least to some extend into thermal energy, which results in a local heating around them. The local heating changes the refractive index that is detected by measuring the scattering of a laser beam.

Non-fluorescent nano objects, e.g. non-fluorescent particles or molecules, mean in the context of the present invention that the nano objects do not or only to a small extent re-emit the energy of the absorbed light in form of or light or electro-magnetic radiation. In particular the non-fluorescent nano objects are characterized by a quantum yield (as the ration of emitted photons per absorbed photons) smaller than 0.15, preferably smaller than 0.05 and mostly preferred smaller than 0.02.

The invention also comprises the use of the device according the invention and absorbing particles or molecules as described above to carry out the method according the invention

The invention is illustrated by the following figures:

Figure 1: Example of a photothermal microscopy setup for twin focus photothermal correlation spectroscopy.

Figure 2: Example of a twin focus photothermal detection volume along the optical axis of a photothermal microscope. The region left of the x-axis denote increased scattering signal and right of the x-axis denote decreased scattering signal and thus a different oscillation phase of the photothermal signal with respect to the phase of the modulated heating laser.

Figure 3: Twin focus photothermal correlation spectroscopy time-trace (right) as well as different exemplary situations of events: (left) motion in one of the focal volume parts or sub-volumes, (second left) directed motion from one to the other volume parts, precisely from one to the other sub-volume.

Figure 4: Example twin focus photothermal correlation functions for different nanoparticle velocities (Vz) along the optical axis. The correlation functions show the temporal auto-correlation of the photothermal signal magnitude (top left), the temporal auto-correlation of the phase sensitive photothermal signal magnitude (top right) and the temporal cross correlation of the positive and negative parts of the phase sensitive signal as depicted in Figure 3.

Figure 5: Dependence of the detection laser beam divergence and the photothermal signal of the position of a heated particle. Thereby, (A) shows a heated particle that is in propagation direction of the detection laser in front of its focal plane, which leads to a narrower beam and an increased photothermal signal. Further, (B) shows a heated particle at the focal plane of the detection laser, which leads to a zero photothermal signal (for non-aberrated laser beams). Finally, (C) shows a heated particle behind the focal plane of the detection laser in its propagation direction, which leads to a wider beam and a decreased photothermal signal.

A detailed scheme of a photothermal microscopy setup for twin-focus photothermal correlation spectroscopy is depicted in Figure 1. The setup has been used to image the twin focus photothermal volume as provided in Figure 2. The twin-focus photothermal signal together with two examples of nanoparticle motion is depicted in Figure 3. Corresponding exemplary temporal correlation functions as obtained from an analysis with the twin-focus photothermal correlation spectroscopy under directed motion of particles along the optical

axis are shown in Figure 4. The influence of the position of a heated particle relative to the focal plane of a detection laser with regard to the beam shape and the relative photothermal signal is depicted in Figure 5 A-C.

The following references are cited within the description of the invention:

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[7] Octeau V, Cognet L, Duchesne L, Lasne D et al. Photothermal absorption correlation spectroscopy. ACS Nano, 3(2):345-50 (2009)

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[9] WO 2006/013272 A2

[10] WO 2004/025278 A1

[11] US 6,756,591 B1

Claims

1. A method of twin-focus photothermal correlation spectroscopy with a focal volume that splits into two sub-volumes forming a twin-focus volume comprising two sharply separated parts of a focal volume showing no spatial overlap.

2. A method according to claim 1 using non-fluorescent absorbing particles or molecules as markers.

3. A method according to claim 1 or 2 detecting phase sensitive signals from the two separated focal volumes.

4. A method according to one of the claims 1 to 3 used to detect anisotropic fluctuations in the concentration of absorbing non-fluorescent markers.

5. A method according to one of the claims 1 to 4 used to detect directed dynamics of absorbing non-fluorescent markers along the optical axis of a photothermal microscope and/or used to detect spatial differences in the fluctuation of absorbing non-fluorescent markers in liquid and biological samples.

6. A method according to one of the claims 1 to 5 including the analysis of the signals obtained by temporal correlation functions, which involve autocorrelation and/or cross correlation of the signals.

7. A method according to one of the claims 1 to 6, whereas depending on the position of a particle or molecule with a divergent gradient index thermal lens around it the detection laser beam will become either wider, narrower or remain unchanged and is thus either decreasing or increasing the detected signal.

8. A method according to one of the claims 1 to 7, whereas the amplitude and phase of the detection laser beam at the modulation frequency of the heating laser is detected and provides the information on the position of the particle or molecule in one of the two separated focal volumes of the twin-focus volume.

9. A method according to one of the claims 1 to 8, whereas amplitude and phase of the detection laser at the modulation frequency of the heating laser is detected to remove

unmodulated contributions when no particle or other non-absorbing objects are in the focus.

10. A device for twin-focus photothermal correlation spectroscopy comprising:

    a.) a heating laser with an intensity preferably modulated at a frequency between 100 kHz and 10 MHz

    b.) a detection laser preferably used in continuous wave (cw) mode,

    whereas both lasers path are merged and focused by a first objective lens to the sample,

    to generate a twin-focus volume both heating and detection laser focal plane overlap, preferably at the same focal point,

    the collected detection laser light is imaged by a second detection or a second microscopy lens on a photo detector or a photodiode.

11. A device according to claim 10 comprising a lock-in amplifier that allows analyzing of the signal of the photo detector or photodiode at the heating laser intensity modulation frequency and to remove unmodulated contributions when no particle or molecule is in the focus.

12. A device according to claim 11, whereas the lock-in amplifier is configured to detect signal amplitude and phase of the detection laser.

13. A device according to claim 12, whereas an axial offset of the focal plane of the heating laser and the focal plane of the detection laser is adjusted by at least one additional lens in front of the objective, with which the divergence of heating and/ or detection laser can be controlled.

14. Use of a device according to one of the claims 11 to 13 to carry out a method according to one of the claims 1 to 9.

15. Use of absorbing particles or molecules as defined in claim 2 to carry out a method according to one of the claims 1 to 9.

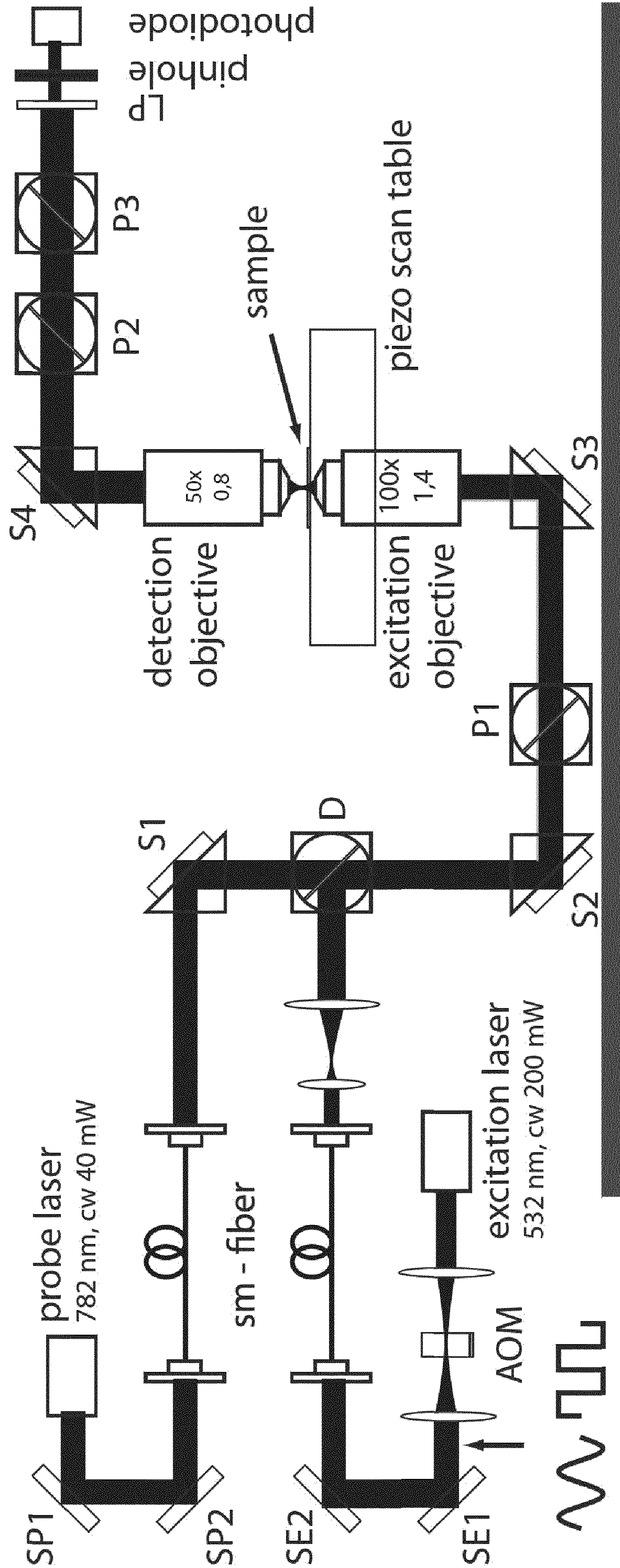

Figure 1

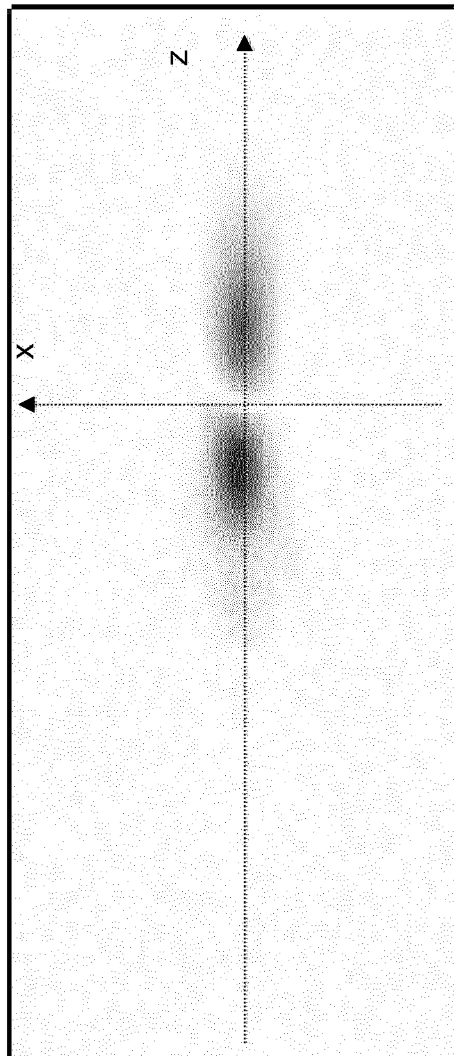

Figure 2

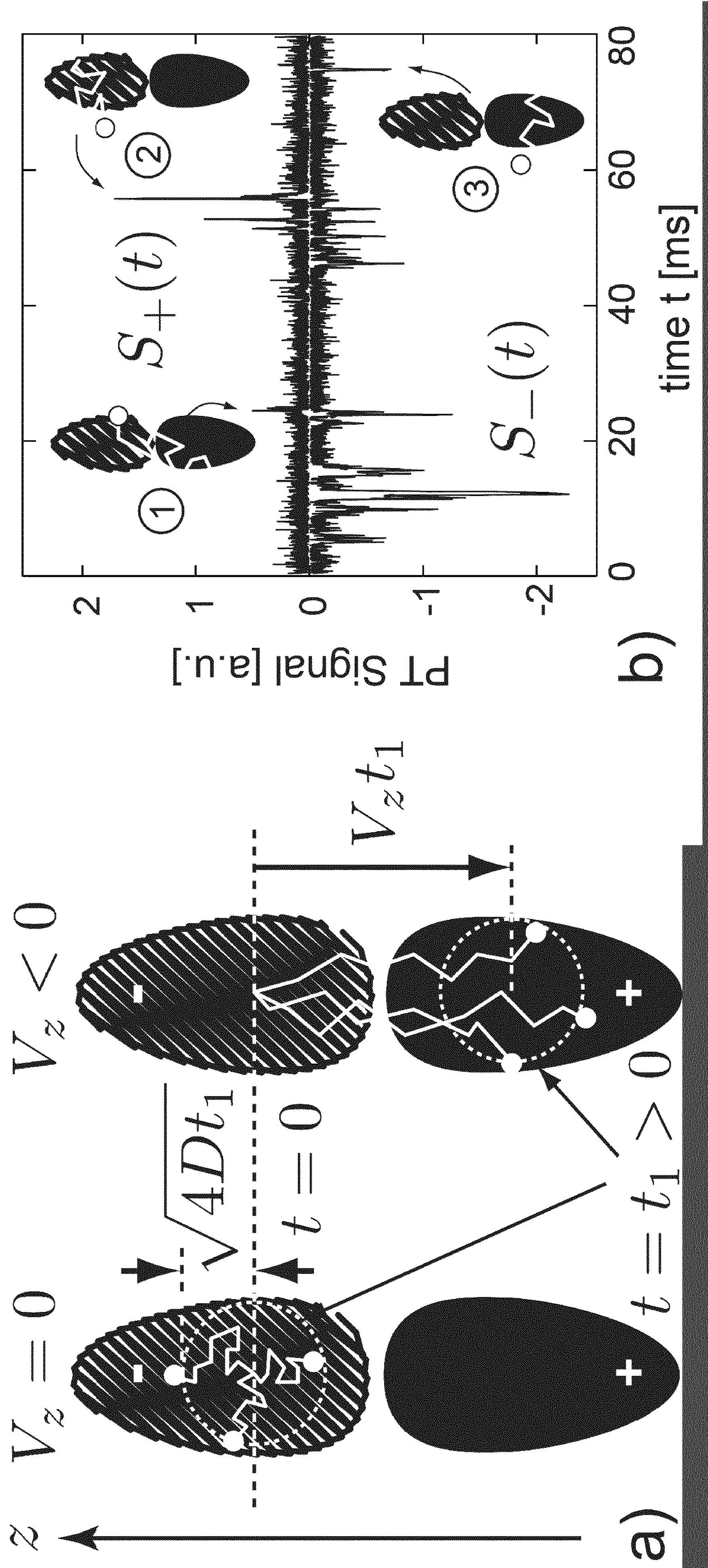

Figure 3

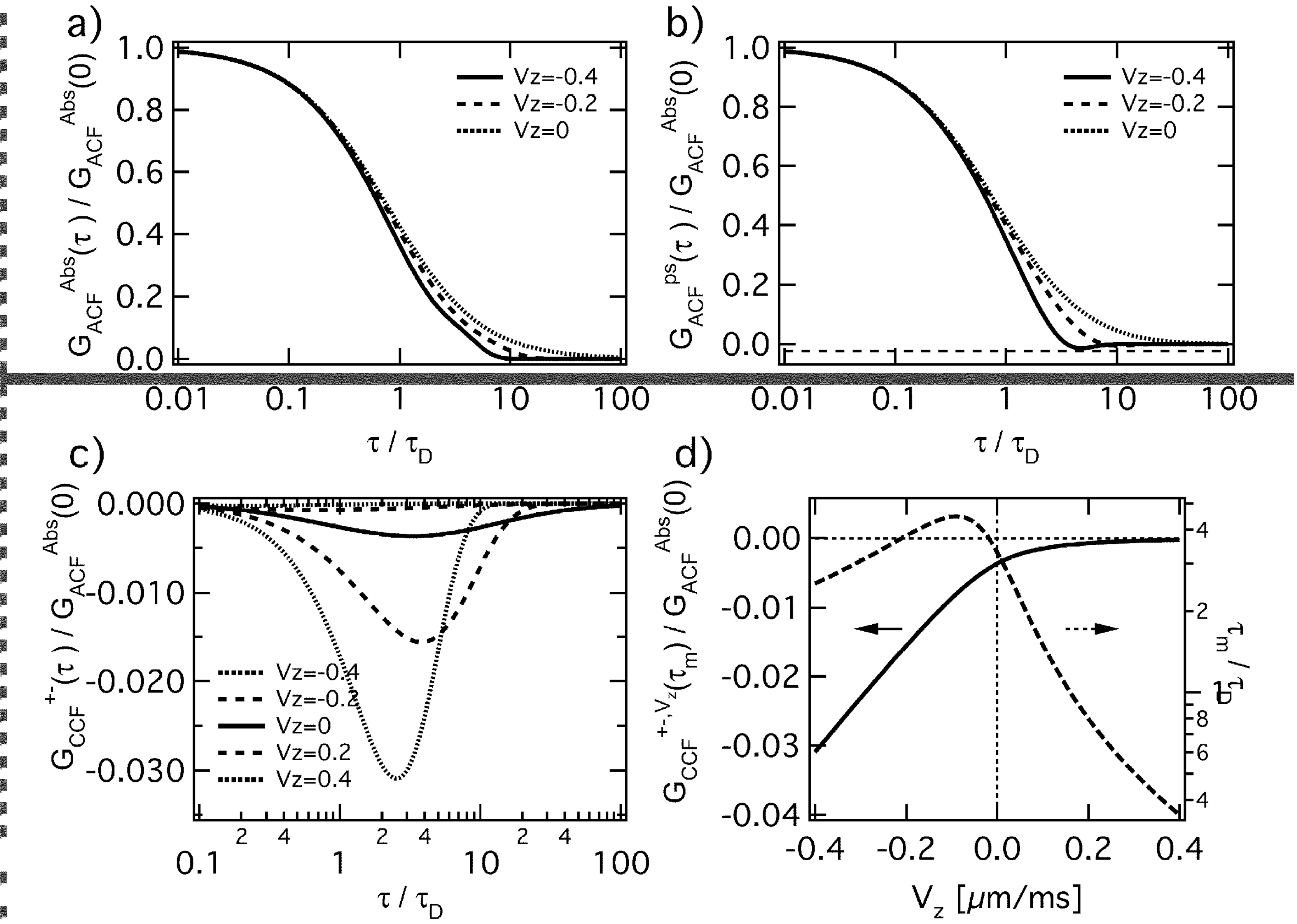

Figure 4

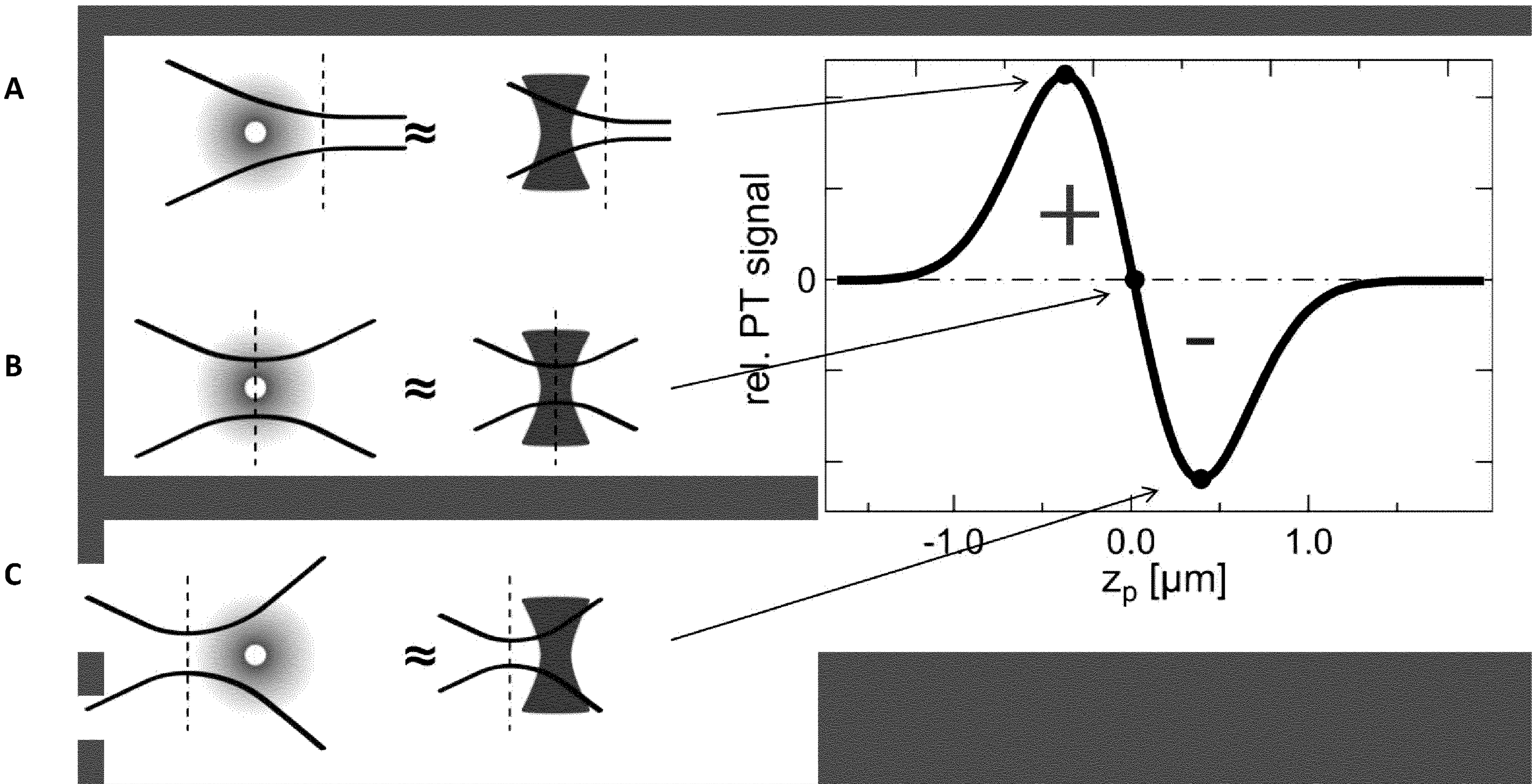

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/063741

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N21/17
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

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, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/150526 A2 (UNIV LEIDEN [NL]; STICHTING TECH WETENSCHAPP [NL]; SPAINK HERMAN PIETE) 17 December 2009 (2009-12-17) figures 1,4 page 4, line 36 - page 6, line 22 page 8, line 16 - line 21 -----	1-15

☐ Further documents are listed in the continuation of Box C.

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Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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