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(54) Title: SIMULTANEOUS HYPERSPECTRAL COHERENT RAMAN MICROSCOPE

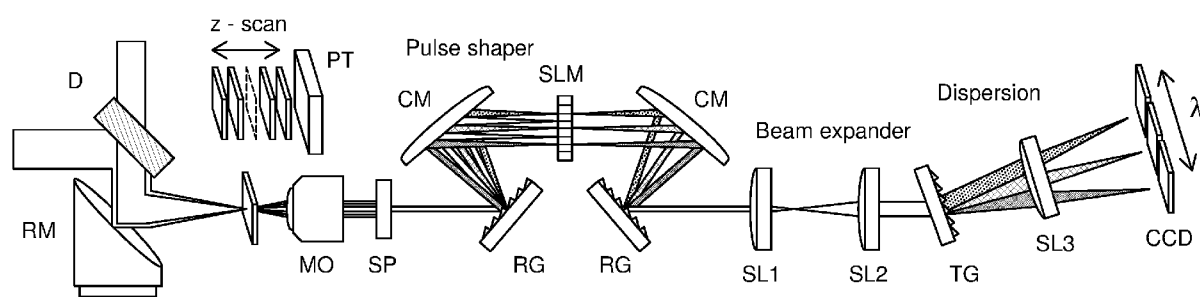


FIG. 1

(57) Abstract: First and second laser devices irradiate a sample in a laser excitation with a laser probe pulse of the second laser device and a time resolved impulsive laser excitation via the first laser device. Coherent Raman signals are generated from the sample over a spatial extent (x- y). Raman signals received from the sample are diffracted via a grating (RG) to a condenser (CM) in order to pass through the tunable spectral filter (SLM) operating in the Fourier plane to keep the size of the spatial dependence minimal. The beam expander (SL1, SL2) and dispersion stage (TG) are arranged for expanding the signal by the beam expander; and propagating through the dispersion stage, such that the spatial dependence become the dominant part in the convolution product at the position of the image sensor (CCD).



Title: Simultaneous Hyperspectral Coherent Raman Microscope

FIELD OF THE INVENTION

5 The invention relates to a method and system for spatially resolved detection of chemical compounds by Raman spectroscopy.

BACKGROUND OF THE INVENTION

Advances in optical imaging techniques over the past decades
10 have revolutionized our ability to study living systems at the microscopic level. Emerging technology for unravelling chemical composition in systems of cellular and molecular biology is needed in biological research and provides a major asset within a wide variety of industrial applications. The requirement for increased complexity and capabilities in multidimensional
15 diagnostic is powered by the search for new biological insight. In order to obtain new biological insight developments involving innovative physical concepts and novel principles within optical spectroscopy and microscopy are essential.

Optical spectroscopy- and microscopy methods have the
20 advantage of being non-invasive, which means that the studied process is not significantly perturbed by the measurement technique. Vibrational imaging techniques offer intrinsic chemical specificity, in that different classes of biomolecules have specific vibrational frequencies serving as unique fingerprints for their identification. Laser based diagnostics may in
25 general provide measurements with exceptionally high spatial- and temporal resolution, which is important in producing reliable and accurate experimental data. Coherent anti-Stokes Raman spectroscopy (CARS) is one such versatile technique, which has had a profound impact on a wide variety of fields. Its unique strengths in molecular specificity and an intense
30 coherent optical signal have enabled study of various physical-, chemical-,

and biological complex systems. Because many complex systems can be fully characterized in multidimensional space, there is a large motivation for the advancement of multidimensional CARS imaging techniques.

In general, when describing a specific optical spectroscopic measurement technique, one can distinguish between the interrogation side and the detection side for the technique. In numerous spectroscopy techniques, based on incoherent (linear) methods, excited light, e.g. from laser induced fluorescence, chemically excited processes (chemiluminescence) and two-photon laser-induced fluorescence, is sent out from molecules typically in isotropic fashion and can be detected in a solid angle arrangement. In other coherent (non-linear) techniques, like CARS for instance, the emission is dictated by a phase-matching condition and is sent out “laser-like” at a specific angle from the sample enhancing the signal throughput.

For the signal generation efficiency and boosting the signal intensity (and also avoiding heat to be deposited on the sample which can easily destroy it), the compilation of the full microscope has the prerequisites of 1. time-resolved impulsive excitation (femtosecond duration laser pulses) and 2. frequency-resolved probing (several hundred femtosecond- to picosecond duration laser pulses) on the interrogation side to generate coherent Raman signals with high intensity.

Many of these previous technologies is performed point-wise, requiring the X-Y image to be compiled through raster scanning of the samples, and for the spectrum in each the pixels conventionally the excitation wavelength is swept.

For example US20100238438 a stimulated Raman scattering image is obtained through raster scanning of the sample. However, since each point in the image plane is sequentially scanned, this is a time consuming process.

Gas-phase spectroscopy has a need for a flexible hyperspectral spectrometer that is much more generic in design and that can image across a wide spectral window (0-4200 cm⁻¹) while handling broad spectral features (~20-200 cm⁻¹) as well.

5 The present invention therefore has as an object to perform spectroscopic broadband interrogation and detection in the wide-field mode, detecting over an entire image area, illuminating and monitoring the entire sample without any rastering procedures.

10 SUMMARY OF THE INVENTION

 The invention aims to achieve this by a method and system of spatially resolved detection of chemical compounds by coherent Raman spectroscopy, comprising providing a sample to be irradiated in a laser excitation that is combined with a laser probe pulse; providing a time
15 resolved impulsive laser excitation of the sample via a first laser pulse; providing a frequency resolved laser probe pulse to the sample via a second laser pulse; said first and second laser pulses being time-synchronized between the excitation and probe pulse; to generate coherent Raman signals from the sample over a spatial extent (x- y) with high intensity across a
20 wide spectral window ranging between 0 and 4200 cm⁻¹.

 According to a further aspect of the invention, the Raman signals are received in a spectral detector, comprised of a pulse shaper, a beam expander; a dispersion stage and image sensor. The pulse shaper is comprised of a grating and collimator pair symmetrically arranged around a
25 tunable spectral filter, in such a way that the Raman signals are diffracted via the grating to the condenser; in order to pass through the tunable spectral filter operating in the Fourier plane to keep the size of the spatial dependence minimal. The signal is expanded by the beam expander; and propagates through the dispersion stage to the image sensor; such that the

spatial dependence becomes the dominant part in the convolution product at the position of the image sensor.

The present invention of a new flexible hyperspectral imager is based on the detection side providing access to relatively fast-dynamics of biochemical processes and monitoring these processes in-vivo at video rate
5 frequencies in relevant biological conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an illustrative embodiment of a snap-shot
10 hyperspectral coherent Raman wide-field microscope.

Figure 2 shows the CARS principles for successful signal generation.

DETAILED DESCRIPTION OF EMBODIMENTS

15 Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs as read in the context of the description and drawings. It will be further understood that terms, such as those defined in commonly used dictionaries, should be
20 interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein. In some instances, detailed descriptions of well-known devices and methods may be omitted so as not to obscure the description of the present systems and methods. The
25 term "and/or" includes any and all combinations of one or more of the associated listed items. It will be further understood that the terms "comprises" and/or "comprising" specify the presence of stated features but do not preclude the presence or addition of one or more other features. All publications, patent applications, patents, and other references mentioned

herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

This hyperspectral coherent Raman microscope is designed for the simultaneous collection of rich data information spanning four dimensions
5 (4D) – (x, y, λ , t). This involves direct spatially-correlated planar interrogation (x-y) acquired with time (t) - and frequency (λ) resolved laser detection methods achieved within a snap-shot “freezing-the-frame”, which is an important condition for a successful diagnostic of cells and organisms in dynamic scenes. This new spectrometer invention is geared towards
10 applications within soft condensed media, with a new unique contrast mechanisms for CARS detection and improved identification of specific biomolecules, and allowing these to be detected in living scenes (Hz video-rate). There are several important systems at target for the proposed instrument, for example the study of stem cell differentiation and formation,
15 metabolism in living cells and organisms, synthesis of high-energy biofuels, and the possibility to probe the transmittance of information through electrical and chemical signals in neural networks.

Turning now to Figure 1 an embodiment is depicted wherein the spectrometer principle is illustrated for simultaneous time- and frequency
20 resolved coherent Raman microscopy achieved over a spatial field. The signal generation plane (x-y) is imaged through a pulse shaper, a beam expander and dispersion optics onto the camera sensor. The full three dimensional spatial sectioning requires a scan along the longitudinal beam propagation direction (z). D – dichroic mirror; RM – reflective mirror; PT –
25 piezoelectric transducer; MO – microscope objective; SP – short pass filter;

Pulse shaper: RG – reflection grating; CM – curved mirror; SLM – spatial light modulator acting as tunable spectral filter operating in the Fourier plane to keep the size of the spatial dependence minimal

Beam expander: SL – spherical lenses;

Dispersion stage: TG – transmission grating;
CCD – camera sensor in the image plane such that the spatial dependence becomes the dominant part in the convolution product at the position of the image sensor. The components of the spectrometer acts as a space-
5 frequency-division multiplexing in the hardware, allowing for independently operating on the space- and the frequency information originating from the interrogated field. The diagnostic can deliver adequate chemical information to decipher the distribution of biomolecules such as lipids, carbohydrates, and proteins in living organisms. The technology is based on broadband
10 excitation (employing ultrashort pulses of sub ($<$) 10 femtoseconds) where the signal is generated across the entire manifold of fundamental Raman active modes, that can be monitored simultaneously. The delay between pump/Stokes- and probe beams can be set arbitrarily and might be different depending on the application. The available excitation bandwidth is directly
15 connected to the temporal pulse-duration of the beam, referred to as near transform-limited pulses or the time-bandwidth product. The shorter the pulse-duration the wider the bandwidth, and vice versa. For ultrashort pulses (sub 10 fs) fundamental Raman active modes can be monitored of most all molecules spanning 0-4200 cm^{-1} . This principle is described e.g. in
20 A. Bohlin et al., Appl. Phys. Lett. 105, 161111 (2014). Broad spectral features can subsequently be filtered and pulse shaped (“narrowed”) before entering the disperser minimizing the convolution between space and frequency at the detector plane of the camera. The broadband spectral peaks are thus being filtered while maintaining the integrity of the spatial
25 information (x-y plane). The grating collimator pairs in the pulse shaper are preferably mounted in 4f-correlator configuration, employed in Littrow condition, in order not to skew the spatial information received from the x-y plane. The novelty lies in the phase matching scheme employed and the optics that allows the dispersed CARS signal from each spatial location in

the plane to be mapped to a CCD detector, along with the novel application to biological systems.

In Fig 2 it is shown how the CARS beam is generated. For successful generation, two physical conditions must be satisfied. One
5 condition is that of energy conservation, where three incident photons with energies ω_{pump} , ω_{Stokes} , and ω_{probe} are mixed with the internal energy levels of the probed molecules to generate a fourth photon at energy ω_{CARS} according to $\omega_{\text{CARS}} = \omega_{\text{pump}} - \omega_{\text{Stokes}} + \omega_{\text{probe}}$. Another condition is phase-matching (momentum conservation), which constrains how the wave vectors of the
10 incident beams must be arranged to effectively generate the Raman shifted coherently scattered light. The microscope can be implemented utilizing the pulse combination two-beam femtosecond/picosecond CARS, which combines highly efficient impulsive excitation in the time domain (pump/Stokes, femtosecond duration laser pulse) with high resolution
15 detection in the frequency domain (probe, several hundred femtosecond- to picosecond duration laser pulse). In the figures, the two broadband femtosecond pulses, i.e. the pump- and the Stokes pulses, are provided by the same laser beam, achieving automatic overlap in time and in space. This is one strong option to go with for the interrogation side considering the
20 intra-pulse configurations, but it is not mandatory.

By phase-locking to an external radio-frequency the laser devices can be phase-locked allowing for arbitrarily arrival step-finesse in the time-synchronization between the excitation and probe pulse. In this way precise electronic timing between the excitation and probe pulse can be achieved
25 which is convenient and beneficial when synchronizing two separate high-power lasers and when the fidelity in time steps do not need to be more than $\sim 5 - 20$ picoseconds (ps) and still be able to delay the relative time arrival between the pulses up to ~ 500 ps, which is applicable working in the gas-phase. In soft condensed phase it is not a critical point, since often the

pump/Stokes- and probe pulses are originating from the same laser and the delay in time arrival between the pump/Stokes pulse and probe pulse is on the order of a few ps (let say 0-5 ps). For example, starting from the output of the ultrafast femtosecond laser, a portion in output power is split off and
5 can become readily engineered; bandwidth compressed to picosecond in pulse duration, shifted in wavelength but still be femtosecond in pulse duration, bandwidth broadened (temporally compressed to sub 10fs), etc. It is beneficial to delay the probe pulse from the pump/Stokes (excitation) pulse to suppress the four-wave-mixing signal with no chemical contrast
10 being generated in the electronic clouds of the molecules (and not the resonant vibrational transitions), referred to as a non-resonant signal. In some applications the non-resonant signal may be used to amplify the resonant signal without smearing the chemical contrast; the skilled person is per se knowledgeable how to engineer the time arrival between the pulses
15 optimizing and balancing between chemical contrast and signal strength. The disclosed simultaneous hyperspectral spectrometer is not dependent on a specific laser pulse configuration.

Throughout the application, any means for carrying out the disclosed methods, in particular, as further clarified below: means imaging, means for
20 splitting, means for relaying can be implemented by optics that are known to the skilled person and may differ in form and structure to arrive at the same function; i.e. the function is physically implemented in optical elements such as mirrors, lenses and prisms. Furthermore, the identified controller functions may be implemented in hardware or software, to
25 provide dedicated processing circuitry that processes input data read from system resources. A server function may e.g. be provided by a connected physical network device, but may also be formed as a virtual device, functioning in a network, and which may be implemented on a hardware resource that can be reached via network communication. These functions

may be executed by one or more processors configured to perform operational acts in accordance with the present systems and methods, such as to provide control signals to the various other module components. The controller may comprise a processor that may be a dedicated processor for performing in accordance with the present system or may be a general-purpose processor wherein only one of many functions operates for performing in accordance with the present system. The processor may operate utilizing a program portion, multiple program segments, or may be a hardware device utilizing a dedicated or multi-purpose integrated circuit.

Any type of processor may be used such as dedicated or shared one. The processor may include micro-controllers, central processing units (CPUs), digital signal processors (DSPs), ASICs, or any other processor(s) or controller(s) such as digital optical devices, or analog electrical circuits that perform the same functions, and employ electronic techniques and architecture. The controller or processor may further comprise a memory that may be part of or operationally coupled to the controller. The memory may be any suitable type of memory where data is stored. Any medium known or developed that can store and/or transmit information suitable for use with the present systems and methods may be used as a memory. The memory may also store user preferences and/or application data accessible by the controller for configuring it to perform operational acts in accordance with the present systems and methods.

The invention is described more fully hereinafter with reference to the accompanying drawings, in which embodiments of the invention are shown.

This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. The description of the exemplary embodiments is

intended to be read in connection with the accompanying drawings, which are to be considered part of the entire written description. In the drawings, the size and relative sizes of systems, components, layers, and regions may be exaggerated for clarity. Embodiments are described with reference to

5 schematic illustrations of possibly idealized and/or intermediate structures of the invention.

Claims

1. A method of spatially resolved detection of a biological substance in an area, comprising:
 - providing a sample to be irradiated in a laser excitation that is combined with a laser probe pulse;
 - 5 – providing a time resolved impulsive laser excitation of the sample via a first laser pulse;
 - providing a frequency resolved laser probe pulse to the sample via a second laser pulse; said first and second laser pulses being time-synchronized; to generate coherent Raman signals from the sample
 - 10 over a wide-field spatial extent (x- y) with high intensity across a wide spectral window ranging between 0 and 4200 cm⁻¹;
 - receiving the Raman signal in a spectral detector, comprised of a pulse shaper, a beam expander; a dispersion stage and a wide-field image sensor;
 - 15 – the pulse shaper comprised of a grating and collimator pair symmetrically arranged around a spectral filter, in such a way that the Raman signals are diffracted via the grating to the condenser; in order to pass through the spectral filter operating in the Fourier plane;
 - 20 – expanding the wide-field signal by the beam expander; and propagating through the dispersion stage to the image sensor in the image plane.
2. A method according to claim 1, wherein the disperser comprises a reflective or transmissive grating arranged to produce a spectrally
- 25 dispersed image at selected wavelengths; selected by the spectral filter.

3. A method according to claim 1, wherein the spectral detector further comprises a microscope objective that can be translated to image a plurality of object planes in z-direction along the incoming beam.
4. A method according to claim 1, wherein the grating collimator pairs in
5 the pulse shaper are mounted in 4f-correlator configuration.
5. A method according to claim 1, wherein said laser excitation and said laser probe pulses are combined via a dichroic mirror D.
6. A method according to claim 1, wherein the beam expander comprises two achromatic lenses.
- 10 7. A system for spatially resolved detection of a biological substance in an area, comprising:
 - first and second laser devices for irradiating a sample in a laser excitation with a laser probe pulse of the second laser device and a time resolved impulsive laser excitation via the first laser device;
 - 15 – a time-synchronizing mechanism for providing a frequency resolved laser probe pulse via the second laser device; said first and second laser devices being synchronized allowing for arbitrarily arrival step-finesse in the time-synchronization between the excitation and probe pulse; to generate coherent Raman signals from the sample over a
20 wide-field spatial extent (x- y) with high intensity across a wide spectral window ranging between 0 and 4200 cm⁻¹;
 - a spectral detector, comprised of a pulse shaper, a beam expander; a dispersion stage and a wide-field image sensor;
 - the pulse shaper comprised of a grating and collimator pair
25 symmetrically arranged around a tunable spectral filter, in such a way that the Raman signals received from the sample are diffracted via the grating to the condenser; in order to pass through the tunable spectral filter operating in the Fourier plane;

- wherein the beam expander and dispersion stage are arranged for expanding the wide-field signal by the beam expander; and propagating through the dispersion stage with the image sensor in an image plane.
- 5 8. A system according to claim 7, wherein the tunable spectral filter comprises a spatial light modulator.

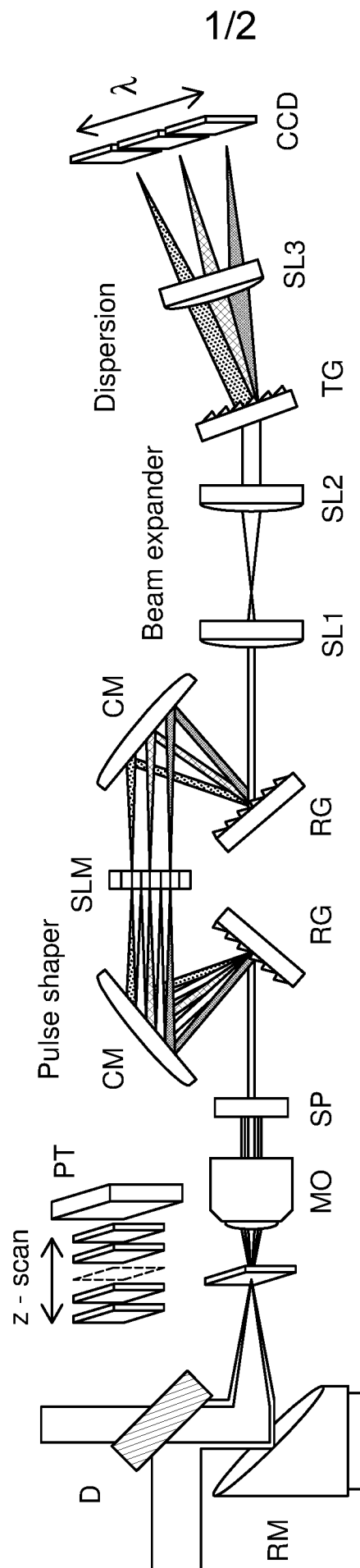


FIG. 1

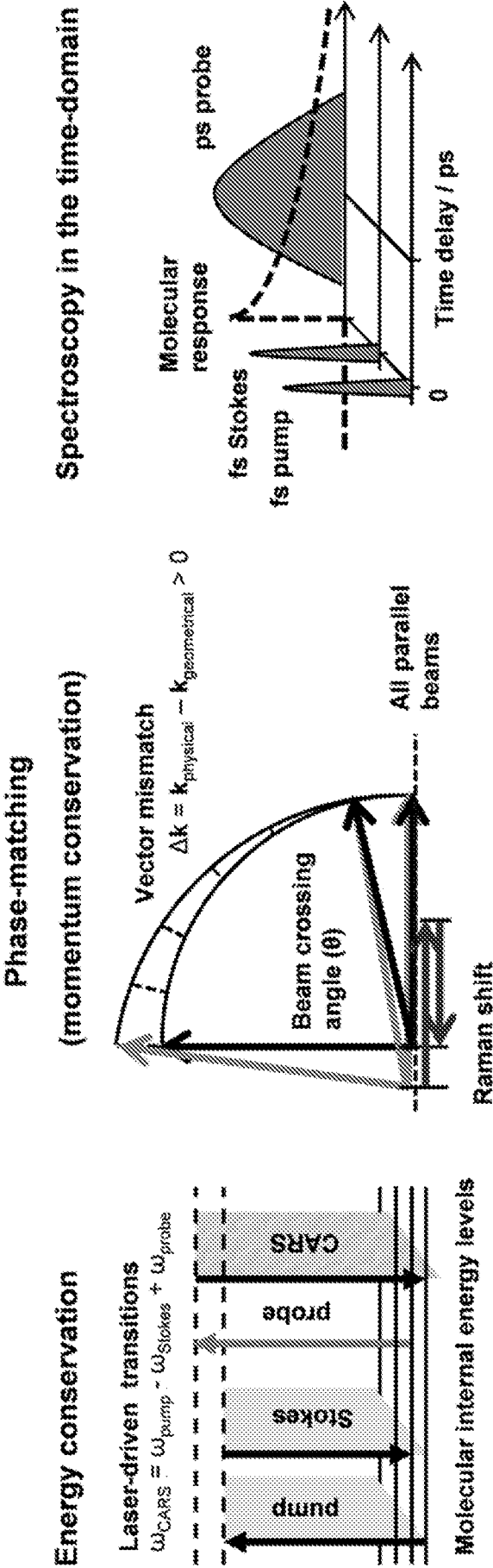


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No
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A. CLASSIFICATION OF SUBJECT MATTER
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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)
G01J 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, WPI Data, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HASHIMOTO ET AL: "High-speed CARS spectral imaging using acousto optic tunable filter", PROCEEDINGS OF THE SPIE,, vol. 7569, 1 January 2010 (2010-01-01), pages 1-7, XP002770101, page 1, paragraph 1 - page 4, paragraph 3; figures 1-6 ----- -/--	1-8



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2017/050596

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MARTIN C. FISCHER ET AL: "<title>Enhancing two-color absorption, self-phase modulation, and Raman microscopy signatures in tissue with femtosecond laser pulse shaping</title>", PROCEEDINGS OF SPIE, vol. 7183, 12 February 2009 (2009-02-12), page 71830V, XP55052331, ISSN: 0277-786X, DOI: 10.1117/12.816488 page 5, paragraph 2; figure 2 -----	1-8
Y	US 2010/238438 A1 (FRANKEL ROBERT D [US]) 23 September 2010 (2010-09-23) cited in the application paragraph [0006] - paragraph [0038]; figures 1-8 -----	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2010238438	A1	23-09-2010	NONE
