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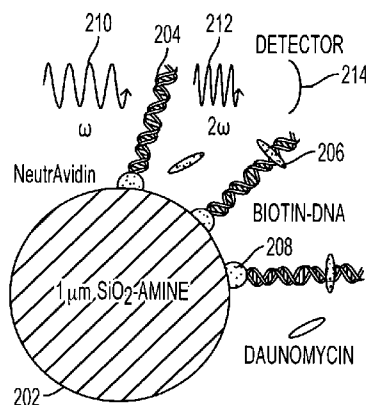


FIG. 2

(57) **Abstract:** Second harmonic generated interference can be used to probe the structure of a molecule. First and second indicator molecules can be bound to a double-stranded oligonucleotide and separated from each other by a number of base pairs. The first and second indicator molecules and the oligonucleotide can be illuminated with an interrogating light, and second-harmonic generated light from the first and second indicator molecules and the oligonucleotide can be detected. A substance that interacts with the oligonucleotide can change the relative orientation of the first and second indicator molecules, resulting in a change in the detected second harmonic generated light. The detected change can be used to determine an effect that the substance has on the structure of the oligonucleotide.

MOLECULE STRUCTURE PROBE METHODS, DEVICES, AND SYSTEMSCROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Application No.

5 61/748,122, filed January 1, 2013, which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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FIELD

The present disclosure relates generally to probing of molecular interactions, and, more
15 particularly, to methods, systems, and devices for probing the structure and interactions of molecules, for example, double-stranded oligonucleotides (*e.g.*, strands of DNA, RNA, etc.) and drugs, using second-harmonic generation (SHG).

SUMMARY

20 The present disclosure describes systems, methods, and devices for probing the structure of at least one target molecule and/or interactions with other molecules, such as drugs, using second harmonic generation are disclosed herein. The embodiments may be used to reveal the structure of target molecules, the structures of complexes of molecules, the time-dependent changes occurring during complex formation or other changes, such as environmental changes
25 and other features. In embodiments, a pair of indicator molecules can be used to generate second harmonic light in response to illumination with interrogating light. Light resulting from the interference of the second harmonic generated light from the pair of indicator molecules is detected, and is indicative of the relative orientation between the pair of indicator molecules. When the pair of indicator molecules is bound to a target molecule (or when each is bound to a
30 separate target molecule), measured changes in the second harmonic generated light pattern can correspond to changes in the target molecule structure that alter the relative orientation of the indicator molecules with respect to each other. This change in structure may be brought about, for example, by interaction of the target molecule with other molecules, such as a drug. Thus, the change in the detected second harmonic generated light can be used to determine an effect
35 that a substance, environmental condition, or other event has on the structure of the target

molecule (or target molecules). The principles may be applied to the study of complexes of molecules, such as double-stranded oligonucleotides (e.g., DNA) and a drug.

In embodiments, a molecule structure probe method can comprise, at a first time, providing first and second indicator molecules bound to a double-stranded oligonucleotide.

- 5 Preferably, the oligonucleotide is formed by intermolecular base pairing (e.g., to form a double-stranded DNA molecule, or a DNA/RNA hybrid), but may also be formed by intramolecular base pairing (e.g., in a single-stranded DNA or RNA molecule).

The first and second indicator molecules can be separated from each other by a number of base pairs. The first and second indicator molecules and the oligonucleotide can be
10 illuminated with an interrogating light, and second-harmonic generated light from the first and second indicator molecules and the oligonucleotide can be detected. A first data profile can be generated based on the detected light. The method can further comprise, at a second time, interacting a substance with the oligonucleotide. After the interacting, the first and second indicator molecules and the oligonucleotide can be further illuminated with the interrogating
15 light, and second-harmonic generated light from the first and second indicator molecules and the oligonucleotide can be detected. A second data profile can be generated based on the detected light. The method can also comprise comparing the first and second data profiles and determining an effect of the substance on the oligonucleotide based on the comparison.

In embodiments, a molecule structure probe method can comprise determining at least
20 one of a shape, an orientation, a spacing, or a relative movement of one or more target molecules or portions thereof responsively to measured second-harmonic generated light emitted from a pair of indicator molecules, which are coupled to the one or more target molecules.

In embodiments, a method for monitoring oligonucleotide structure can comprise using a pair of molecules intercalated into a double-stranded oligonucleotide and detecting changes to
25 the oligonucleotide structure by detecting second-harmonic generated interference from the pair of molecules during a structure change-inducing action.

In embodiments, a method can quantify the shape, orientation, spacing, or relative movement between first and second target molecule portions, which are portions of a single target molecule or respective portions of two separate target molecules that are interacting, for
30 example, by complexing. The method can comprise providing a light source, a light detector, and a processor. The method can further comprise binding a first indicator molecule to the first target molecule portion and binding a second indicator molecule to the second target molecule portion. The method can also comprise, using the light source, generating a non-linear optical effect responsive to the relative spacing and/or orientation between the first and second indicator
35 molecules. The method can further comprise, using the light detector, measuring the non-linear

optical effect and predicting therefrom, information indicative of the shape, orientation, spacing, or relative movement between the first and second target molecule portions.

In embodiments, a system for probing one or more target molecules having a pair of indicator molecules coupled thereto can comprise an interrogating light source, a detector, and a controller. The detector can be configured to detect second-harmonic generated (SHG) light emitted by the one or more target molecules and to generate a signal responsively to the detected SHG light. The controller can be coupled to the light source and the detector. The controller can be configured to control the light source to illuminate the molecule and to receive the generated signal from the detector. The controller can be further configured to determine a relative orientation of the pair of indicator molecules based on the generated signal.

Objects and advantages of embodiments of the disclosed subject matter will become apparent from the following description when considered in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF DRAWINGS

Embodiments will hereinafter be described with reference to the accompanying drawings, which have not necessarily been drawn to scale. Where applicable, some features may not be illustrated to assist in the illustration and description of underlying features. Throughout the figures, like reference numerals denote like elements.

FIG. 1 is a diagram showing various features of a system for probing molecules, according to one or more embodiments of the disclosed subject matter.

FIG. 2 illustrates probing of a microparticle coated with DNA strands having intercalated daunomycin molecules, according to one or more embodiments of the disclosed subject matter.

FIG. 3 shows the chemical structure of daunomycin, according to one or more embodiments of the disclosed subject matter.

FIG. 4 shows a single strand of DNA with a pair of intercalated daunomycin molecules, according to one or more embodiments of the disclosed subject matter.

FIG. 5 is a schematic depiction of DNA duplexes affixed on a spherical particle, with the inset showing a representation of the induced second harmonic dipole moments, $\mathbf{p}_A$ and $\mathbf{p}_B$, of two identical molecules, A and B, respectively, in the molecule-pair principal coordinate system defined by $\{x', y', z'\}$ with relative orientation angle of $\Phi = 2\gamma$, according to one or more embodiments of the disclosed subject matter.

FIG. 6 is a graph of representative binding isotherms collected with different spacing (20, 17, or 15 base pairs, listed on the right, corresponding to SEQ ID NOs:2, 3, and 4,

respectively) between a pair of daunomycin molecules bound to DNA, according to one or more embodiments of the disclosed subject matter.

FIG. 7 is a graph of measured second harmonic intensity versus base pair separation (lower axis) and orientation angle (upper axis), where the relative orientation of the bound daunomycin molecules is shown as arrows associated with each data point, according to one or more embodiments of the disclosed subject matter.

FIG. 8 shows the relative angles between generated second harmonic fields in the coordinate frame discussed herein, according to one or more embodiments of the disclosed subject matter.

FIGS. 9A-9B illustrate a target molecule with a pair of indicator molecules before and after interacting with a substance, according to one or more embodiments of the disclosed subject matter.

FIG. 10 is a process flow diagram of a method for probing a double-stranded oligonucleotide based on second harmonic generated interference, according to one or more embodiments of the disclosed subject matter.

FIGS. 11A-11B illustrate multiple target molecules, each with an indicator molecule, before and after a disturbance that changes the relative orientation of the indicator molecules, according to one or more embodiments of the disclosed subject matter.

FIG. 12 is a process flow diagram of a method for probing a molecule based on second harmonic generated interference, according to one or more embodiments of the disclosed subject matter.

DETAILED DESCRIPTION

Second Harmonic Generation (SHG) can be used to probe microscale changes in the structure of a target molecule or target molecules, for example, oligonucleotides exposed to a drug, protein, peptide, or other substance or condition that may affect its structure. SHG is a nonlinear optical process whereby photons with the same frequency (ω) combine to form new photons with twice the frequency (2ω) due to interaction with a particular medium, e.g., one or more indicator molecules that are coupled to one or more target molecules.

FIG. 1 illustrates components of a system 100 that can be used to probe a target molecule according to embodiments of the disclosed subject matter. A target molecule or molecules can be disposed in a sample chamber 102, for example, a cuvette or other light transparent sample holder. A light source 104 can illuminate the sample chamber 102 and the target molecules therein with radiation of a particular frequency (ω). The light source 104 can be, for example, a laser, such as a pulsed Ti:Sapphire laser or any other suitable source of light. The light source

104 may generate coherent light, for example, having a wavelength in the visible or infrared portions of the electromagnetic spectrum.

Interaction of the light from the source with the target molecules and/or indicator molecules can generate second harmonic (SH) light, which has a frequency of 2ω . Detector 106
5 can be arranged to detect the light emitted from the sample chamber 102. Detector 106 can include an optical system, for example, to focus the light from the sample chamber 102 to an appropriate detecting medium, such as a CCD or other photodetector. Detector 106 can also include other optical processing components, for example, to select for desired wavelengths, such as a spectrograph or optical notch filters.

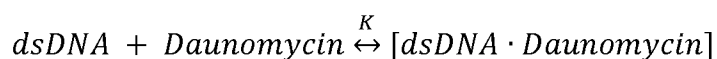
10 A control system 108 can be coupled to the source 104 and the detector 106 for coordinating operation thereof (i.e., simultaneous illumination and detection or sequential illumination and detection). Control system 108 may also include a processor configured to determine properties of the target molecule or target molecules based on the SH light detected by the detector 106. For example, the processor of control system 108 may be configured with
15 computer-readable instructions that cause the processor automatically to determine a shape or change in shape of one or more target molecules responsively to a signal from the detector 106, which signal corresponds to the SH light detected by the detector 106. Memory 114 can be coupled to the control system 108 for storing acquired data, for example, as a reference in later determinations. The system 100 can further include input/output device 112 and/or display 110
20 to allow a user to interact with the control system 108 and to view results of the detection.

SHG can be used to determine equilibrium binding constants for biomolecular interactions in a label free and noninvasive way. For example, SHG can be used to probe the binding of a drug to DNA tethered to the surface of colloidal microparticles suspended in aqueous solution. Biomolecule coatings on nano- and microparticle surfaces can allow for self-
25 assembling of the superstructured material. Such a configuration builds on interface specific nonlinear optical experiments that have measured the electronic and vibrational spectra of DNA covalently bound to planar fused quartz surfaces immersed in aqueous media, and experiments that tracked in real time the cleavage of DNA by the restriction enzyme EcoR1 and the subsequent rehybridization of DNA attached to colloidal polymer microparticles without labeled
30 reporter molecules or invasive detection methods.

Using SHG to study biomolecular reactions, with one of the reactants attached to a colloidal interface, can yield larger signals than analogous experiments performed at planar interfaces due to the larger number of molecules that can be accommodated in the laser focus. Additionally, the number of particles in solution can be changed without changing the density of

the DNA that is attached to the individual particles, allowing for flexibility in experimental design.

Daunomycin (structure shown in FIG. 3) is a member of the class of anthracycline chemotherapeutic drugs that is used to treat acute leukemia. It is generally accepted that the anticancer properties arise from the intercalation of the aromatic rings of daunomycin into the DNA double helix while simultaneously stabilizing the complex through H-bonding of its amino sugar in the DNA minor groove. To establish the mechanism by which a drug functions for use in drug design, it is necessary to determine the equilibrium binding constant, K , of the drug to a target receptor site and to extract the related thermodynamic properties to elucidate the inherent driving forces. For the binding of daunomycin to double stranded DNA (dsDNA), the relevant reaction is given by:



In drug-DNA binding experiments, a sequence of dsDNA can be designed such that it contained one “recognition sequence” or “recognition site,” which is a sequence of three base pairs that are known to preferentially bind daunomycin. For example, daunomycin most favorably binds to 5'-(TCG)-3', (ACG), (AGC), and (TGC) triplet sequences. More generally, this indicates that daunomycin favors adjacent GC nucleotides as binding sites. As crystal structures have shown, it is at the location of these bases that the fused rings of daunomycin are intercalated into the DNA double helix. A DNA sequence that can be used in the recognition experiments is given by:

biotin-5'-CTCAAGTGAAGTCAAGTGAATCCAATTCGAAGTT-3', (SEQ ID NO:1) with the 5'-TCG-3' recognition sequence bolded and underlined. The complementary strand is implied for succinctness. The control dsDNA sequence can differ by replacing the cytosine and guanine bases of the TCG recognition sequence with thymidine and cytosine bases, respectively, i.e., by substituting the TCG recognition sequence with TTC such that adjacent GC bases were intentionally replaced with TC. Persons of ordinary skill in the art will recognize that a double-stranded nucleotide sequence other than that represented by SEQ ID NO:1 could also be used, so long as it contains at least one daunomycin recognition sequence. FIG. 2 is a schematic diagram of a DNA-coated microparticle 202 and an exemplary reaction/detection scheme. A plurality of DNA strands 204, for example biotin-DNA, can be tethered to microparticle 202 by a biomolecule 208. Although only three strands of DNA are shown in FIG. 2, any number of strands of DNA may be bound to the microparticle 202. Microparticle 202 can be a microsphere, for example, a silica-amine (SiO₂-amine) microbead. The microsphere can have a diameter on the order of microns, for example, 100µm or less, such as

10µm or less. For example, the microsphere can have a diameter of 1µm or less. The biomolecule 208 can be, for example, NEUTRAVIDIN® (avidin, Life Technologies), which is a deglycosylated version of avidin. Daunomycin molecules 206 can intercalate into one or more of the DNA strands 204, as illustrated in FIG. 2.

5 Incident electromagnetic radiation 210 at a frequency, ω , interacts with molecules (e.g., DNA strand 204 and daunomycin 206) and induces a polarization that oscillates at the incident frequency and at multiple orders of the incident frequency. Of interest are those interactions that generate a coherent second order polarization at twice the fundamental laser frequency, 2ω , which is SHG 212 that can be detected by detector 214. Coherent signals originating from the
10 second order polarization, which include sum frequency, difference frequency, and second harmonic generation, are generally forbidden in centrosymmetric and isotropic media. Although coherent SHG is forbidden in centrosymmetric and isotropic bulk media for reasons of symmetry, it can be generated by a centrosymmetric structure, e.g., a sphere, provided that the object is centrosymmetric over roughly the length scale of the optical coherence, which is a
15 function of the particle size, the wavelength of the incident light, and the refractive indices at ω and 2ω .

The radiated SHG field, $E_{2\omega}$, from an individual particle is proportional to the second order polarization, $P_{2\omega}$, at 2ω and is given by:

$$E_{2\omega} \propto P_{2\omega} = \chi_T E_\omega E_\omega \quad (1)$$

where the total second order susceptibility, χ_T , is the sum of the second order susceptibilities of
20 all adsorbed species on an individual particle and E_ω is the incident electromagnetic field. The second order susceptibility for adsorbate species i can be written as:

$$\chi_i \propto N_i \sum_{k,e} \frac{\mu_{gk} \mu_{ke} \mu_{eg}}{(\omega_{gk} - \omega - i\Gamma)(\omega_{eg} - 2\omega - i\Gamma)} \quad (2)$$

where μ_{gk} , μ_{ke} , and μ_{eg} are the transition dipole matrix elements between electronic states of the molecules, ω_{gk} and ω_{eg} are the transition frequencies between states, Γ is the line width for the transitions, and N_i is the number density of the i th species at the interface of an individual
25 particle. Eqn. 2 illustrates that when an electronic transition of an interfacial molecule is resonant with ω or 2ω , there is an enhancement of the SHG signal. Since daunomycin has an absorption tail at $2\omega = 420$ nm, it dominates the SHG response.

At a low density of particles, e.g., 2.5×10^8 particles/mL, the SHG field from each particle is independent of other particles and has random phases. The total SHG intensity,

$I_{2\omega}(\text{total})$, is an incoherent summation of the intensity generated by each particle, $I_{2\omega}$, and can be given by:

$$I_{2\omega}(\text{total}) \propto \sum_j^n |E_{2\omega}|^2(j) = nI_{2\omega} \quad (3)$$

where n is the number density of particles and $|E_{2\omega}|^2(j)$ is the absolute square of the radiated SHG field from the j th particle.

5 It is the changing magnitude of the SHG signal from daunomycin as it binds to dsDNA attached to the silica-amine beads that tracks the binding. In the absence of daunomycin, the SHG intensity is due to nonresonant SHG from the microparticle and incoherent SHG, i.e., hyper-Rayleigh scattering (HRS), which arises from density and orientation fluctuations of the bulk molecules. Bulk water can largely be responsible for the observed HRS background signal.
10 If the interface is charged, there can be an additional source of SHG radiation originating from the third order polarization, $P_{2\omega}$, oscillating at 2ω .

The contribution to the SHG signal from the third order polarization is described as a product of two oscillating electric fields and an electrostatic, zero frequency field that extends from the interface into the bulk media and polarizes the bulk molecules (predominantly water
15 molecules). The magnitude of the signal from the third order polarization can be strongly dependent on the pH and the electrolyte concentration of the solution, which serve to neutralize charges at the interface and to screen bulk molecules from the electric field generated by the charged interface. Experiments were carried out in a relatively concentrated electrolytic solution (e.g., 50 mM Tris buffer) such that the contribution from the third order polarization was
20 negligible.

To probe the binding of daunomycin 206 to dsDNA 204 attached to colloidal microparticles 202, a second harmonic generation apparatus was employed. An interrogating light source (i.e., source 104 illustrated in FIG. 1) was used to illuminate the dsDNA sample. In experiments, the interrogating light source was a Ti:Sapphire oscillator running at a repetition
25 rate of 80 MHz, with a center wavelength of 840 nm, producing approximately 300 mW average power with a pulse width of 60 fs. However, other illuminating light sources are also possible according to one or more contemplated embodiments.

Interrogating light from the source was focused into a 2 mm quartz cuvette containing the sample. The generated SHG radiation was collected with a short focal length lens,
30 telescoped, and filtered to remove much of the residual fundamental laser radiation before it was focused to a detector 214 (e.g., detector 106 illustrated in FIG. 1). The detector can include one or more optical systems (e.g., for focusing) and/or one or more optical processing systems (e.g.,

for filtering or separating light into a frequency spectrum). In experiments, the detector included a spectrograph (e.g., an Acton SpectraPro 300i spectrograph) coupled to an imaging device (e.g., a Princeton Instruments Spec-10 CCD camera). The camera exposure was set to 1 second such that several hundred exposures could be collected per concentration step to acquire statistics.

- 5 Measurements were reproduced at least three times and averaged to establish reproducibility and improve error bars. Quoted uncertainties are given at the 95% confidence level.

In experiments, NEUTRAVIDIN® (avidin) was covalently attached to the surface of 1 μm diameter silica-amine microparticles (from Polysciences, Inc.). The positively charged silica-amine particles help to reduce electrostatic binding of positively charged daunomycin to the surface of the particle. A 33-mer of biotinylated dsDNA (SEQ ID NO:1, from Integrated DNA Technologies) was dissolved in 50 mM Tris buffer at pH 7.5, and added to the solution containing the particles. The DNA surface coverage on the microparticles was estimated to be ~1.6 × 10⁴ DNA/μm², as determined from particle centrifugation measurements. The concentration of the microparticles used in experiments was 2.5 × 10⁸ particles/mL, which yielded sufficient signal while minimizing scattering losses. The reactions were carried out in 50 mM Tris buffer at pH 7.5. The bulk concentration of daunomycin (Sigma-Aldrich) was determined from optical absorption measurements using the extinction coefficient at λ = 480 nm, ε₄₈₀ = 11500 M⁻¹ cm^{-1.68}.

The SHG intensity is proportional to the square of the number of interfacial daunomycin-dsDNA complexes. A good fit of the data is obtained by utilizing the Langmuir binding model

$$I_{2\omega}(total) \propto \left[\frac{Kc}{(1 + Kc)} \right]^2 \quad (4)$$

where $I_{2\omega}(total)$ is the observed SHG intensity that changes as the available binding sites on DNA are filled, K is the equilibrium constant for the reaction of daunomycin with dsDNA, and c is the bulk concentration of daunomycin in solution. From the measurements of the SHG signal as a function of daunomycin concentration, the equilibrium constant of daunomycin binding to the 33-mer of DNA having a specific recognition sequence can be obtained using Eqn. 3.

Based on experiments, the SHG binding isotherm fit with the relationship in Eqn. 3 yields an equilibrium binding constant of $2.3 (\pm 0.7) \times 10^5 \text{ M}^{-1}$, and thereby a Gibbs free energy of $-7.2 \pm 0.2 \text{ kcal/mol}$ at 20 °C. The presence of excess DNA in solution was found to not influence the binding isotherms, since the concentration of DNA is sufficiently low that there is negligible depletion of bulk daunomycin. To establish that daunomycin binds specifically to the recognition site in the dsDNA, and that the measured equilibrium constant describes the

propensity of daunomycin to this sequence, control experiments were performed where the three base pairs of the recognition sequence were replaced with TTC bases, such that the control dsDNA does not contain adjacent GC nucleotides. The results of the control experiments show that daunomycin does not bind to the control DNA, as expected, since the control DNA does not include the preferential daunomycin intercalation sites. Therefore, the equilibrium binding constants measured in the recognition DNA experiments describes the binding of daunomycin to the TCG sequence. This also implies that only one daunomycin is bound to each recognition DNA duplex.

The selectivity of SHG provides a high sensitivity to the population of daunomycin-DNA complexes relative to linear optical methods because only the drug-DNA complexes at the microparticle interface generate coherent SHG signals. In linear optical methods, all of the absorbing species in solution contribute to the signal, which includes the free daunomycin, daunomycin aggregates, and the daunomycin-DNA complexes, and must be addressed in the data analysis. The SHG binding isotherms selectively monitor the drug-DNA complexes at the microparticle interface and are straightforward to construct and analyze using simple models to describe the binding equilibrium.

The ability to monitor an indicator molecule bound to a target molecule (e.g., daunomycin intercalated in a DNA strand) using second harmonic generation can be extended to provide details regarding changes in the structure of the target molecule (e.g., winding, unwinding, or bending of the DNA strand). In embodiments of the disclosed subject matter, the relative spatial orientation of two indicator molecules bound to a target molecule (e.g., DNA duplex) can be systematically manipulated and subsequently probed using second harmonic (SH) generation (SHG).

For example, as illustrated in configuration 400 in FIG. 4, one daunomycin molecule 404 (having orientation 406) can be rotated relative to another daunomycin molecule 408 (having orientation 410) by changing the number of base pairs between two recognition sites on a given DNA duplex 402 that was affixed to a colloidal microparticle. Each DNA base pair on translation up or down the helix 402 to the location of the adjacent base pair imparts a 36° rotation to the helical DNA structure. Thus, the relative orientation 406, 410 of DNA intercalators 404, 408 with defined recognition sites can be controlled by simply changing the number of bases pairs separating them on a given DNA duplex.

Although daunomycin is specifically discussed herein, other indicator molecules are also possible according to one or more contemplated embodiments. Indeed molecules capable of repeatable arrangement in a target molecule (e.g., DNA), that is not readily displaced from the target molecule, and that exhibits relatively strong SHG for a given input light wavelength can

be used. For example, the indicator molecules can have all electronic transitions substantially in a single plane. When the target molecule is DNA, the indicator molecule can intercalate with its plane substantially perpendicular to the helical axis of the DNA. Preferably, the indicator molecule is one that can intercalate between nucleic acid base pairs. In addition, although
 5 examples with a pair of indicator molecules are discussed, the use of more than two indicator molecules per target molecule or target molecule portion is also possible according to one or more contemplated embodiments.

The interference of SH fields generated from the intercalated species can be exploited in order to map the measured SH intensity to the intercalated molecules' relative orientation. In
 10 experiments, the interference of radiated SH fields generated from bound daunomycin molecules on a DNA duplex was measured and found to be modulated by the relative spatial orientation of the two molecules. The interference of the SH radiation can be described in a simple model framework discussed below. Taking advantage of the tailored interference, embodiments of the disclosed subject matter can serve as a solution phase "molecular protractor" to measure DNA
 15 unwinding angles for a broad range of protein-DNA and drug-DNA complexes. Moreover, embodiments of the disclosed subject matter can more generally quantify the shape, orientation, spacing, or relative movement between first and second target molecule portions, which can be portions of a single molecule or respective portions of molecules that are interacting.

SHG is a coherent surface specific spectroscopy where intense light at frequency ω
 20 induces a second order polarization that oscillates at twice the driving frequency, and thereby generates light at twice the fundamental frequency, 2ω . Since SHG is coherent, the radiated SH intensity from a single particle is given by

$$I_{2\omega} \propto \left[\sum_i E_{2\omega}^i \right]^2 \quad (5)$$

where $E_{2\omega}^i$ is the SH field from a given interfacial molecule, which add up, with its respective phase, with other interfacial molecules to produce the overall SH intensity from an individual
 25 particle.

In experiments, the total measured SHG intensity is the incoherent sum of the coherent SH light generated by the daunomycin pair bound to the DNA on the individual particles in solution. The isotropic bulk medium does not produce coherent SH radiation since the SH field generated by one randomly oriented molecule is, in principle, canceled out by another molecule
 30 that has the opposite orientation, leading to an overall destructive interference of the bulk SH signal. However, incoherent SH light can be generated in the bulk medium by density and orientational fluctuations of the species in bulk solution. In experiments, the observed SH

intensity generated by a pair of molecules bound to DNA, whose orientations are well defined and controlled, is shown to contain information on the relative orientation of the two molecules, allowing for detailed solution phase investigations of relative molecular orientation.

In experiments, the second harmonic generation apparatus described above was employed. Thus, the interrogating light source was a Ti:Sapphire oscillator running at a repetition rate of 80 MHz, with a center wavelength of 840 nm, producing approximately 300 mW average power with a pulse width of 60 fs. Interrogating light from the source was focused into a 2 mm quartz cuvette containing the sample. The generated SHG radiation was collected with a short focal length lens, telescoped, and filtered to remove much of the residual fundamental laser radiation before it was focused to a detector, which included a spectrograph (e.g., an Acton SpectraPro 300i spectrograph) coupled to an imaging device (e.g., a Princeton Instruments Spec-10 CCD camera). The camera exposure was set to 1 second such that several hundred exposures could be collected per concentration step to acquire statistics. Measurements were reproduced at least three times and averaged to establish reproducibility and improve error bars. Quoted uncertainties are given at the 95% confidence level.

Biotinylated-DNA (Integrated DNA Technologies) was coupled to 1 μ m NEUTRAVIDIN® (avidin) coated silica-amine beads. The total concentration of DNA was 420 nM, to ensure complete surface coverage. The particle density was 2.5×10^8 particles/mL, and the samples were suspended in 50 mM Tris buffer at pH = 7.5. The SH intensity measured from the beads and excess DNA in solution (i.e., not attached to the particle) was the same as neat water, within experimental uncertainty. Excess DNA in solution does not influence the binding isotherms.

In experiments, the specificity of daunomycin to the sequence TCG was used to spatially orient two intercalated daunomycin molecules on a given DNA duplex. Daunomycin has the effect of lengthening the DNA helical axis by approximately 3.4 Å, and imparting a small unwinding of the DNA helix (approximately 8°) relative to non-complexed B-DNA. In experiments, the sequence of the DNA 33-mer duplex was referenced to the sequence used where only one recognition site was present:

biotin-5'-CTC AAG TGA ACT CAA GTG AAT CCA ATC GAA GTT-3' (SEQ ID NO:1), with the recognition site highlighted (complementary strand is implied). To change the orientation angle between the two drugs on a DNA duplex, the distance between two recognition sites was varied by changing the number of base pairs separating the two recognition sites. For example, the DNA used where the spacing was 20 bases apart was:

biotin-5'-CTC AAT CGA ACT CAA GTG AAT CCA ATC GAA GTT-3' (SEQ ID NO:2).

Persons of ordinary skill in the art will recognize that a double-stranded nucleotide sequence other than that represented by SEQ ID NO:2 could also be used with the methods of the instant disclosure, so long as it contains at least two recognition sites for an indicator molecule. Such a double-stranded sequence may be represented by the following formula (I):

$$5 \qquad A_x-D_1-B_y-D_2-C_z \qquad (I)$$

where:

A, B, and C signify a nucleotide base pair selected individually for each of A, B, and C independently of one another,

10 D_1 and D_2 signify first and second indicator molecule recognition sites (respectively), selected individually for each and independently of one another, and preferably being selected from the group consisting of 5'-TCG-3', 5'-ACG-3', 5'-AGC-3', and 5'-TGC-3' (with the complementary strand of each implied, for brevity),

x and z signify an integer selected individually for each and independently of one another, from 1 to 3, 1 to 4, 1 to 5, 1 to 6, 1 to 7, 1 to 8, or 1 to 9, preferably from 15 4 to 6, and most preferably 5, and

y signifies an integer from 5 to 30, 10 to 25, 10 to 20, 10 to 17, 10 to 15, and preferably 10, 12, 14, or 17.

Persons of ordinary skill in the art will also recognize that a double-stranded nucleotide sequence other than that represented by SEQ ID NO:1 could also be used with the methods of the instant disclosure, so long as it contains at least one recognition site for an indicator molecule. Such a double-stranded sequence may be represented by the following formula (II):

$$A_x-D-B_y \qquad (II)$$

where A, B, and x, have the same meanings as those given for formula (I) above, where D signifies an indicator molecule recognition site, preferably being selected from the group consisting of 5'-TCG-3', 5'-ACG-3', 5'-AGC-3', and 5'-TGC-3' (with the complementary strand of each implied, for brevity), and where y signifies an integer from 10 to 30, 15 to 25, 20 to 15, and preferably 25.

Persons of ordinary skill in the art will recognize that formulae (I) and (II) encompass various species of double-stranded nucleotide sequences and will further recognize that different species may be combined with one another for use with the methods of the instant disclosure (e.g., different species of formula (I), with same or different indicator molecule recognition sites, used together; different species of formula (II), with same or different indicator molecule recognition sites, used together; and/or different species (I and II), with same or different indicator molecule recognition sites, used together).

Preferably, the nucleotide sequence is at least 75% homologous and preferably at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% homologous to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:2.

FIG. 5 shows a system 500 with multiple DNA duplexes 502 affixed on a spherical particle 504. The relative orientation of two intercalating nonlinear polarizable molecules (denoted as arrows 506 and 508) bound to each DNA 502 indicate the directions of the induced SH dipole moments. The inset shows a schematic representation of the induced SH dipole moments $\mathbf{p}_A$ and $\mathbf{p}_B$ of two identical molecules, A and B, respectively, in the molecule-pair principal coordinate system defined by $\{x', y', z'\}$ with relative orientation angle of $\Phi = 2\gamma$.

Representative binding isotherms collected in the experiment are shown in FIG. 6. As illustrated in FIG. 6, the SH intensity at the maximum density of bound daunomycin, which is the plateau of the isotherm, changes as the spacing between the two recognition sequences was varied. The daunomycin intercalates with its aromatic plane oriented at right-angles to the axis of the DNA helix. The orientation angle between the two daunomycin molecules is given by $\Phi = n * 36^\circ$, where n is the number of base pairs separating the two recognition sites. Therefore, when $n = 5, 15, 25$, etc., the intercalated molecules at the two binding sites are anti-parallel and the SHG will destructively interfere. Similarly, when $n = 10, 20, 30$, etc., the intercalated molecules at the two binding sites are aligned parallel and will constructively interfere, as observed from the SH binding data in FIG. 6.

To quantify the modulated SHG intensity, the signal intensity at the isotherm plateau was averaged and plotted versus the number of base pairs separating the two binding sites in FIG. 7 (20 bp separation = SEQ ID NO:2; 17 bp separation = SEQ ID NO:3; 15 bp separation = SEQ ID NO:4; 13 bp separation = SEQ ID NO:5). This plot more clearly illustrates the oscillatory behavior of the resulting SH light intensity that results from systematically rotating one daunomycin molecule relative to the other, i.e., by changing the number of base pairs separating the pair of daunomycin molecules. The relative orientation is depicted in FIG. 7 as arrows representing the individual daunomycin molecules.

A simple model can be used to quantify the interference of radiated SH fields from systematically oriented daunomycin molecules on a DNA duplex. A coordinate system is defined according to FIG. 8 where two generated SH fields, E_a and E_b are defined relative to the x-axis by the angles ϕ_a and ϕ_b for E_a and E_b , respectively. Similarly, the angle out of the x-y plane is defined as θ_a and θ_b . The SH intensity is the coherent sum of the individual SH fields and can be written as:

$$I_{2\omega} \propto |\mathbf{E}_a + \mathbf{E}_b|^2 = |\mathbf{E}_a|^2 + |\mathbf{E}_b|^2 + 2\mathbf{E}_a \cdot \mathbf{E}_b \quad (6)$$

where, each SH field can be written explicitly in terms of vector components to give:

$$\mathbf{E}_a = E_a(\sin\theta_a\cos\phi_a\hat{\mathbf{x}} + \sin\theta_a\sin\phi_a\hat{\mathbf{y}} + \cos\theta_a\hat{\mathbf{z}}) \quad (7)$$

The DNA's helical axis is defined along the z-axis in this coordinate system so the DNA bases lie parallel to the x-y plane. Based on the crystal structure of the daunomycin-DNA complex, daunomycin is known to intercalate parallel to the DNA's bases yielding the approximation:

$$\theta_a \approx \theta_b \approx 90^\circ.$$

5 Applying this approximation to Eqn. 7 and substituting into Eqn. 6 yields:

$$I_{2\omega} \propto |\mathbf{E}_a|^2 + |\mathbf{E}_b|^2 + 2|\mathbf{E}_a||\mathbf{E}_b|(\cos\phi_a\cos\phi_b + \sin\phi_a\sin\phi_b) \quad (8)$$

Simplifying and noting that the intensity is proportional to the orientational average of the intercalated daunomycin molecules gives:

$$I_{2\omega} \propto |\mathbf{E}_a|^2 + |\mathbf{E}_b|^2 + 2|\mathbf{E}_a||\mathbf{E}_b|\langle\cos(\phi_a - \phi_b)\rangle \quad (9)$$

Setting $\Phi = \phi_a - \phi_b$ and noting that $|\mathbf{E}_a| = |\mathbf{E}_b| \equiv |E_{2\omega}|$ gives:

$$I_{2\omega} \propto 2|E_{2\omega}|^2[1 + \langle\cos\Phi\rangle] \quad (10)$$

10 where $|E_{2\omega}|^2$ physically represents the generated SH intensity of one daunomycin molecule bound to DNA. In order to fit the experimental data, the orientational average above is replaced with an empirical parameter, g , that can range from 0 to 1 to account for imperfect SH interference, yielding:

$$I_{2\omega} \propto 2|E_{2\omega}|^2[1 + g \cdot \cos(\Phi)] \quad (11)$$

The experimental data in FIG. 7 was fit to Eqn. 7 to yield:

$$g = 0.53 \pm 0.17, \text{ and}$$

$$|E_{2\omega}|^2 = 101 \pm 15 \text{ arb. units.}$$

15 The value of $|E_{2\omega}|^2$ extracted from the fit is in agreement with the SH intensity that was observed in experiments with one recognition site (i.e., 120 ± 17 arb. units). The agreement between the fit and the measured intensity from the binding of daunomycin to one recognition site serves as an internal consistency check to ensure the model and fit retrieve meaningful parameters.

20 The retrieved value of g indicates that the interference "efficiency" is ~53%. The deviation from ideal SH interference might be due to the ensemble distribution of orientations

that the two molecules can take relative to one another, resulting in imperfect orientation. Additionally, the daunomycin molecules might be twisted or tilted slightly from one another when intercalated into the DNA, which is not explicitly accounted for here. The slight unwinding of DNA by daunomycin could lead to imperfect interference of the SHG fields, which would be observed in FIG. 7 as a phase shift (i.e., the maxima and minima would not coincide with 0° or 180° , respectively). Since no obvious shift is observed in the data shown in FIG. 7 and the data is well described by Eqn. 11, the unwinding induced by daunomycin on DNA can be neglected. Despite the simplicity and the approximations made, the model successfully recovered the correct oscillation frequency of the SH intensity versus base pair separation, i.e., the frequency was not freely fit and reproduced the known periodicity of the DNA double helix (36° per base pair).

The disclosed approach to control and measure molecular orientation presents opportunities to probe structural aspects of bio-molecular complexes in solution. For example, it is possible to investigate the unwinding of DNA in various protein or drug complexes since the relative orientation and SH intensity response act as a “molecular protractor” that echoes changes to the DNA helix upon complex formation. The interference of the SH electric fields generated by the pair of SH-active chromophores (e.g., daunomycin) bound to DNA is strongly dependent on their relative orientation. Structural changes induced by foreign molecules binding to DNA will change the relative orientation of the two chromophores and thereby change the SH interference pattern. Any distortion to the helical twist angle of DNA induced by another binding ligand in between two chromophores can be recovered by measuring the change in SH intensity. Moreover, the DNA remains label free in the region where the DNA duplex complexes with foreign molecules such as drugs, proteins, peptides, etc.

With the disclosed SH interference method, winding can be differentiated from the unwinding of a DNA duplex by a relatively simple change in the interference pattern. A modulated reference pattern can be obtained in the absence of any binding moiety from measurements of the SH intensity as the number of base pairs separating the two bound daunomycin molecules is increased or decreased. For example, a DNA strand 902 can have a first daunomycin molecule 904 at a first location with a first orientation (indicated by the arrow) and a second daunomycin molecule 906 at a second location with a second orientation (indicated by the arrow), as shown in FIG. 9A. A translation of daunomycin by a single base pair, up or down the helix, imparts a rotation of 36° for daunomycin with respect to the helical axis. Thus the number of base pairs separating the daunomycin molecules 904, 906 determines their relative orientation.

The recognition site of a substance 908 (e.g., a drug, protein, peptide, etc.) can be placed between the two daunomycin molecules 904, 906 as shown in FIG. 9B. For example, the substance 908 can be separated from each daunomycin molecule 904, 906 by a minimum number of base pairs, for example, at least ten base pairs. Each daunomycin molecule 904, 906 can be separated from the other daunomycin molecule by a maximum number of base pairs, for example, no more than thirty base pairs. Moreover, the daunomycin molecules 904, 906 can be positioned along the DNA helix 902 away from ends thereof, for example, at least ten base pairs, to avoid significantly affecting the structure of the DNA 902.

The nature of the perturbation effected by the binding of the substance 908 to the DNA structure 902 determines whether there is increased winding, unwinding, or bending of the DNA helix, thereby resulting in a change in orientation of the indicator molecules 904, 906 and a corresponding change in the measured SHG interference. For example, the anti-viral and antibiotic drug netropsin complexes with DNA by minor groove binding, which results in an increased winding of the DNA helix. The increased winding can thus be manifested by a shift and higher frequency interference pattern with respect to the interference pattern of the reference sample (e.g., FIG. 9A without substance 908).

If there is an increased winding, as is the case for the binding of netropsin, then the increase in the twist per base pair, would result in the modulation in the SH signal occurring at a smaller number of base pairs separating the daunomycin molecules. For the case when unwinding is the result of a drug binding to DNA, the shift in the interference pattern would be in the opposite direction, i.e., towards an increased number of base pairs separating the daunomycin molecules. In addition to information of the structural change in DNA (i.e., winding, unwinding, etc.), the magnitude of the shift can provide an indication of the degree of winding/unwinding.

Referring to FIG. 10, a process flow diagram of an exemplary method for probing the effect of a substance on the structure of a double-stranded oligonucleotide, such as a strand of DNA, is shown. The process can begin at 1002 where the oligonucleotide is tethered to a colloidal microsphere, such as, but not limited to, a silica-amine microbead, as shown in FIGS. 2 and 5. At 1004, a first indicator molecule is intercalated at a first position of the oligonucleotide. At 1006, a second indicator molecule is intercalated at a second position of the oligonucleotide. Although described separately, steps 1004 and 1006 can occur simultaneously, for example, by allowing the indicator molecules in solution to bind to respective sites on the oligonucleotide, such as described above with respect to daunomycin and DNA. The arrangement of the first and second indicator molecules may depend, for example, on the selection of base pairs for the oligonucleotide, and, more particularly, on the base pairs as the

desired binding sites. The first and second indicator molecules may be arranged, for example, so as to be separated by at least 10 base pairs but no more than 30 base pairs of the oligonucleotide.

At 1008, the oligonucleotide can be illuminated with interrogating radiation, which can be light having a wavelength in the visible or infrared portion of the electromagnetic spectrum. At 1010, light emanating from the illuminated oligonucleotide can be detected. In particular, the second harmonic light generated by the indicator molecules, which may constructively or destructively interfere depending on their relative orientation, is detected, for example, by using a spectrograph and an appropriate detector, such as a CCD or other photodetector. Although described separately steps 1008 and 1010 can occur simultaneously, for example, by measuring light concurrent with illumination and filtering the illumination from the detected light. Alternatively, steps 1008 and 1010 can occur sequentially, such that detection is only active after an applied light pulse illuminates the oligonucleotide.

At 1012, the detected SHG interference can be used to determine a first relative orientation of the first and second indicator molecules. This first relative orientation may be saved as a reference orientation by which subsequent changes to the first and second indicator molecules and/or the oligonucleotide structure can be measured. At 1014, the oligonucleotide is exposed to a test condition, which may impact the structure of the nucleotide. For example, the oligonucleotide may be exposed to a drug, peptide, protein, or any other substance. Alternatively or additionally, the oligonucleotide can be exposed to a particular condition, such as temperature change, pressure change, time change, gravitational change or any other condition, which may affect the structure of the oligonucleotide. The oligonucleotide can be structured such that the interaction with the substance is between the first and second indicator molecules, as illustrated in FIG. 9B.

At 1016, the oligonucleotide can be re-illuminated with interrogating radiation, which can be light having a wavelength in the visible or infrared portion of the electromagnetic spectrum. At 1018, light emanating from the illuminated oligonucleotide can again be detected. In particular, the second harmonic light generated by the indicator molecules, which may constructively or destructively interfere depending on their relative orientation, is detected, for example, by using a spectrograph and an appropriate detector, such as a CCD or other photodetector. Although described separately steps 1016 and 1018 can occur simultaneously, for example, by measuring light concurrent with illumination and filtering the illumination from the detected light. Alternatively, steps 1016 and 1018 can occur sequentially, such that detection is only active after an applied light pulse illuminates the oligonucleotide.

At 1020, the detected SHG interference from the re-illumination can be used to determine a second relative orientation of the first and second indicator molecules. This second relative orientation can be compared to the reference orientation at 1022. At 1024, the change in the oligonucleotide (e.g., winding, unwinding, bending, kinking, etc.) due to exposure to the test condition can be determined based on the change in the orientation (i.e., from the first relative orientation to the second relative orientation) of the indicator molecules.

Embodiments of the disclosed subject matter are not limited to a single oligonucleotide. Rather, the disclosed methods and systems can be applied to multiple target molecules or portions thereof. Referring to FIGS. 11A-11B, a first target molecule 1102 and a second target molecule 1104 are shown. The first target molecule 1102 may be interacting with the second target molecule 1104 at an interaction region 1110. Each target molecule may have a respective indicator molecule, for example, indicator molecule 1108 for target molecule 1104 and indicator molecule 1106 for target molecule 1102. At a first time, the indicator molecules can have a first relative orientation. For example, as shown in FIG. 11A, the orientation of indicator molecule 1106 can be opposite to that of indicator molecule 1108, as indicated by the arrows. However, at a second time, when one or more of the target molecules undergoes a structural change, such as due to exposure to a drug or other substance, the orientation may change. For example, as shown in FIG. 11B, the orientation of indicator molecule 1108 becomes the same as indicator molecule 1106 due to condition 1112 changing the arrangement of target molecule 1104. This change in orientation can be probed by a corresponding change in the detected SHG from the first time to the second time.

FIG. 12 is a process flow diagram of an exemplary method for more generally probing the structure of a target molecule or target molecules. The process can begin at 1202, where a first indicator molecule is coupled at a first location. At 1204, a second indicator molecule is coupled at a second location. Although described separately, steps 1202 and 1204 can occur simultaneously, for example, by allowing the indicator molecules in solution to bind to respective sites on the oligonucleotide, such as described above with respect to daunomycin and DNA. Moreover, as discussed above, the first and second indicator molecules may be coupled to different portions of the same target molecule or different target molecules.

At 1206, the indicator molecules can be illuminated with interrogating radiation, which can be light having a wavelength in the visible or infrared portion of the electromagnetic spectrum. At 1208, light emanating from the illuminated molecules can be detected. In particular, the second harmonic light generated by the indicator molecules, which may constructively or destructively interfere depending on their relative orientation, is detected, for example, by using a spectrograph and an appropriate detector, such as a CCD or other

photodetector. Although described separately, steps 1206 and 1208 can occur simultaneously, for example, by measuring light concurrent with illumination and filtering the illumination from the detected light. Alternatively, steps 1206 and 1208 can occur sequentially, such that detection is only active after an applied light pulse illuminates the molecules.

5 At 1210, the detected SHG interference can be used to determine a first relative orientation of the first and second indicator molecules. Optionally, the process can be repeated, for example, with the first and second indicator molecules at different locations. For example, the process can be repeated with the first and second indicator molecules at various base pair spacings, so as to acquire data similar to that shown in the graph of FIG. 7. Alternatively or
10 additionally, the process can be repeated after exposure to a condition that may alter the structure or arrangement of the one or more target molecules. For example, the process can be repeated after exposing the target molecule to a drug or other substance, as discussed above with respect to FIGS. 9A-9B.

A measurement system according to the above experimental apparatus description may
15 be provided with a light source, filters, receiver, etc. and a processor to receive, process, and output the data according to the above described techniques, which may be readily translated by those skilled into computer based processing steps capable of outputting the changes in the shape, orientation, spacing, or relative movement between the first and second target molecule portions, for example, the daunomycin molecules. It will be apparent that other target molecules
20 and binding devices other than intercalation may be used to exploit the principals of the disclosed embodiments.

Many anti-cancer drugs bind to DNA, but they can unwind the double helix thereby changing the degree of rotation per nucleotide. To gain a full molecular understanding of DNA-binding drug action, this drug-induced unwinding can be measured using embodiments of the
25 disclosed subject matter, i.e., the relative orientation of a pair of DNA-binding molecules can be measured using SHG. The information acquired using such embodiments can be used to extract the extent of DNA unwinding or other changes (e.g., winding, kinking, supercoiling, bending, etc.) on the DNA structure induced by interaction with the drug. In contrast to conventional structural techniques such as crystallography, embodiments of the disclosed subject matter are
30 able to acquire information with the drug in solution thereby more closely mimicking the DNA environment *in vivo*. The information regarding drug-DNA interaction obtained using embodiments of the disclosed subject matter could be useful in designing more effective anti-cancer and other drugs.

In first embodiments, a molecule structure probe method comprises, at a first time,
35 providing first and second indicator molecules bound to a double-stranded oligonucleotide. The

first and second indicator molecules are separated from each other by a number of base pairs. The molecule structure probe method at the first time can further comprise illuminating the first and second indicator molecules and the oligonucleotide with an interrogating light, and detecting second-harmonic generated light from the first and second indicator molecules and the oligonucleotide. The molecule structure probe method at the first time can further comprise generating a first data profile based on the detected light.

In first embodiments, the molecule structure probe method can comprise, at a second time, interacting a substance with the oligonucleotide, and after the interacting, further illuminating the first and second indicator molecules and the oligonucleotide with the interrogating light. The molecule structure probe method at the second time can further comprise detecting second-harmonic generated light from the first and second indicator molecules and the oligonucleotide and generating a second data profile based on the detected light. The molecule structure probe method can further comprise comparing the first and second data profiles and determining an effect of the substance on the oligonucleotide based on the comparison.

In first embodiments or any other embodiments, the second harmonic light can be sampled over time to obtain a time-resolved record of a process, for example, complexation of first and second molecules. The first and second molecules can be according to any of the examples described herein.

In first embodiments or any other embodiments, the determining an effect comprises quantifying a shape of the oligonucleotide.

In first embodiments or any other embodiments, the determining an effect comprises quantifying an amount of winding or unwinding of the oligonucleotide.

In first embodiments or any other embodiments, the determining an effect comprises quantifying an amount of bending of the oligonucleotide.

In first embodiments or any other embodiments, the first and second indicator molecules are chromophores.

In first embodiments or any other embodiments, each of the first and second indicator molecules is a planar molecule having its respective electronic transitions in a single plane that is oriented perpendicular to a helical axis of the oligonucleotide.

In first embodiments or any other embodiments, the first and second indicator molecules are separated by at least ten base pairs.

In first embodiments or any other embodiments, the first and second indicator molecules are separated by no more than thirty base pairs.

In first embodiments or any other embodiments, the first and second indicator molecules are intercalated into the oligonucleotide.

In first embodiments or any other embodiments, the first and second indicator molecules comprise daunomycin.

5 In first embodiments or any other embodiments, the first and second indicator molecules are intercalating nonlinear polarizable molecules.

In first embodiments or any other embodiments, the substance is at least one of a drug, a protein, or a peptide.

10 In first embodiments or any other embodiments, the oligonucleotide is bound to a spherical particle.

In first embodiments or any other embodiments, the oligonucleotide is bound to a colloidal silica microparticle.

In first embodiments or any other embodiments, the oligonucleotide is bound to a silica-amine microparticle having a diameter of 1 μm or less.

15 In first embodiments or any other embodiments, the interrogating light has a wavelength of 840nm.

In first embodiments or any other embodiments, the interrogating light has a frequency of ω and the second-harmonic generated light has a frequency of 2ω .

20 In second embodiments, a molecule structure probe method comprises determining at least one of a shape, an orientation, a spacing, or a relative movement of one or more target molecules or portions thereof responsively to measured second-harmonic generated light emitted from a pair of indicator molecules, which are coupled to the one or more target molecules.

25 In second embodiments or any other embodiments, the emitted second-harmonic generated light is due to illumination of the indicator molecules with interrogating light, and the second-harmonic generated light has a frequency twice that of the interrogating light.

In second embodiments or any other embodiments, the interrogating light has a wavelength of 840nm.

In second embodiments or any other embodiments, the one or more target molecules comprise a double-stranded oligonucleotide.

30 In second embodiments or any other embodiments, the indicator molecules are intercalated in the oligonucleotide.

In second embodiments or any other embodiments, the indicator molecules are separated from each other along the oligonucleotide by no more than thirty base pairs.

In second embodiments or any other embodiments, a number of base pairs between the pair of indicator molecules define the relative orientation of one of the indicator molecules with respect to the other.

5 In second embodiments or any other embodiments, the indicator molecules comprise daunomycin.

In second embodiments or any other embodiments, each indicator molecule is coupled to a different portion of a target molecule or a different target molecule from the other indicator molecule.

10 In second embodiments or any other embodiments, each indicator molecule is a planar molecule having electronic transitions in a single plane.

In second embodiments or any other embodiments, the one or more target molecules are tethered to one or more colloidal microparticles suspended in aqueous solution.

In second embodiments or any other embodiments, each microparticle comprises a silica amine microsphere.

15 In second embodiments or any other embodiments, each microsphere has a diameter of 1 μm or less.

In third embodiments, a method for monitoring oligonucleotide structure comprises using a pair of molecules intercalated into a double-stranded oligonucleotide to measure changes to the oligonucleotide structure based on second-harmonic generated interference from said pair
20 of molecules.

In third embodiments or any other embodiments, the intercalated molecules comprise daunomycin.

In third embodiments or any other embodiments, the measured changes comprise a winding or unwinding of the oligonucleotide.

25 In third embodiments or any other embodiments, the method comprises exposing the oligonucleotide to a drug, protein, or peptide, the measured changes being a result of said exposing.

In third embodiments or any other embodiments, the exposing results in a winding, unwinding, or bending of the oligonucleotide.

30 In third embodiments or any other embodiments, the second-harmonic generated interference is produced by illuminating the intercalated molecules with visible or infrared light.

In third embodiments or any other embodiments, the illuminating light has a wavelength of 840nm.

35 In fourth embodiments, a method can quantify the shape, orientation, spacing, or relative movement between first and second target molecule portions. The target molecule portions are

portions of a single target molecule or respective portions of two separate target molecules that are interacting. The method can comprise providing a light source, a light detector, and a processor. The method can further comprise binding a first indicator molecule to the first target molecule portion and binding a second indicator molecule to the second target molecule portion.

5 The method can also comprise, using the light source, generating a non-linear optical effect responsive to the relative spacing and/or orientation between the first and second indicator molecules, and, using the light detector, measuring the non-linear optical effect. The method can further include predicting from the measured non-linear optical effect information indicative of the shape, orientation, spacing, or relative movement between the first and second target
10 molecule portions.

In fourth embodiments or any other embodiments, the method further comprises outputting said information from said processor.

In fourth embodiments or any other embodiments, the binding comprises intercalating the first indicator molecule to the first target molecule portion and intercalating the second
15 indicator molecule to the second target molecule portion.

In fourth embodiments or any other embodiments, the non-linear light comprises second harmonically generated light.

In fourth embodiments or any other embodiments, the single target molecule or the two separate target molecules comprise drugs.

20 In fifth embodiments, a system is configured to perform any of the methods disclosed herein.

In sixth embodiments, a system can probe one or more target molecules having a pair of indicator molecules coupled thereto. The system can comprise an interrogating light source, a detector, and a controller. The detector is configured to detect second-harmonic generated
25 (SHG) light emitted by the one or more target molecules and to generate a signal responsively to the detected SHG light. The controller is coupled to the light source and the detector. The controller is configured to control the light source to illuminate the molecule and to receive the generated signal from the detector. The controller is further configured to determine a relative orientation of the pair of indicator molecules based on the generated signal.

30 In sixth embodiments or any other embodiments, the interrogating light source comprises a pulsed laser.

In sixth embodiments or any other embodiments, the laser is a Ti:Sapphire laser.

In sixth embodiments or any other embodiments, the interrogating light source produces visible or infrared light.

In sixth embodiments or any other embodiments, the interrogating light source produces light having a wavelength of 840nm.

In sixth embodiments or any other embodiments, the detector comprises a CCD camera.

In sixth embodiments or any other embodiments, the detector comprises a spectrograph.

5 In sixth embodiments or any other embodiments, the detector is configured to detect emitted light having a frequency twice that of light from the interrogating light source.

In sixth embodiments or any other embodiments, the system further comprises a memory module configured to store the determined relative orientation of the pair of indicator molecules.

10 In sixth embodiments or any other embodiments, the controller is further configured to determine a change in the one or more target molecules based on a comparison of a determined relative orientation with a previously stored relative orientation.

In sixth embodiments or any other embodiments, the system further comprises a microsphere to which the one or more target molecules are tethered.

15 In sixth embodiments or any other embodiments, the microsphere is a silica amine microsphere having a diameter of 1 μ m or less.

In sixth embodiments or any other embodiments, the one or more target molecules comprises a double-stranded oligonucleotide, and the controller is configured to determine an amount of winding, unwinding, or bending of the oligonucleotide responsively to the determined relative orientation.

20 In any of the disclosed embodiments, the SHG interference from a pair of indicator molecules can be used as an assay to test for interaction of a particular agent with one or more target molecules. For example, if a certain biological agent interacts with a known portion of a double-stranded oligonucleotide, the pair of indicator molecules can be arranged on the oligonucleotide with the known portion therebetween. The construct of the indicator molecules
25 with the oligonucleotide can then be used as an assay to detect for the presence of the biological agent, i.e., by interaction of the biological agent with the known portion to alter the structure of the oligonucleotide which thereby changes the SHG from the indicator molecules.

In any of the disclosed embodiments, the SHG interference from a pair of indicator molecules can be used to screen libraries of molecules or compounds to find ones that interact
30 with particular portions of an oligonucleotide. For example, the pair of indicator molecules can be arranged on the oligonucleotide with a known sequence therebetween. The construct of the indicator molecules with the oligonucleotide can then be exposed to a particular compound. Changes in the measured SHG from the indicator molecules can be indicative of the particular compound interacting with the known sequence of the oligonucleotide.

In any of the disclosed embodiments, the SHG interference from a pair of indicator molecules can be used to determine what sequence a particular known compound interacts with. For example, multiple DNA fragments with different sequences, each bracketed by a pair of indicator molecules can be used. The known compound can be sequentially exposed to each DNA fragment, wherein the measured SHG from the pair of indicator molecules can provide an indication of the DNA fragment with which the known compound interacts.

In any of the disclosed embodiments, the target molecule can be any kind of paired nucleic acid structure, such as DNA/RNA hybrids, a DNA structure interacting with itself (e.g., intra-molecular interaction), or RNA structures (e.g., stem-loop structures, etc.).

It will be appreciated that the modules, processes, systems, and devices described above can be implemented in hardware, hardware programmed by software, software instruction stored on a non-transitory computer readable medium or a combination of the above. For example, a method for probing the structure of a molecule using second harmonic generated light can be implemented, for example, using a processor configured to execute a sequence of programmed instructions stored on a non-transitory computer readable medium. For example, the processor can include, but is not limited to, a personal computer or workstation or other such computing system that includes a processor, microprocessor, microcontroller device, or is comprised of control logic including integrated circuits such as, for example, an Application Specific Integrated Circuit (ASIC). The instructions can be compiled from source code instructions provided in accordance with a programming language such as Java, C++, C#.net or the like. The instructions can also comprise code and data objects provided in accordance with, for example, the Visual Basic™ language, LabVIEW, or another structured or object-oriented programming language. The sequence of programmed instructions and data associated therewith can be stored in a non-transitory computer-readable medium such as a computer memory or storage device which may be any suitable memory apparatus, such as, but not limited to read-only memory (ROM), programmable read-only memory (PROM), electrically erasable programmable read-only memory (EEPROM), random-access memory (RAM), flash memory, disk drive and the like.

Furthermore, the modules, processes, systems, and devices can be implemented as a single processor or as a distributed processor. Further, it should be appreciated that the steps mentioned herein may be performed on a single or distributed processor (single and/or multi-core). Also, the processes, modules, and sub-modules described in the various figures of and for embodiments herein may be distributed across multiple computers or systems or may be co-located in a single processor or system. Exemplary structural embodiment alternatives suitable for implementing the modules, systems, or processes described herein are provided below.

The modules, processes, systems, and devices described above can be implemented as a programmed general purpose computer, an electronic device programmed with microcode, a hard-wired analog logic circuit, software stored on a computer-readable medium or signal, an optical computing device, a networked system of electronic and/or optical devices, a special purpose computing device, an integrated circuit device, a semiconductor chip, and a software module or object stored on a computer-readable medium or signal, for example.

Embodiments of the methods, processes, modules, devices, and systems (or their sub-components or modules), may be implemented on a general-purpose computer, a special-purpose computer, a programmed microprocessor or microcontroller and peripheral integrated circuit element, an ASIC or other integrated circuit, a digital signal processor, a hardwired electronic or logic circuit such as a discrete element circuit, a programmed logic circuit such as a programmable logic device (PLD), programmable logic array (PLA), field-programmable gate array (FPGA), programmable array logic (PAL) device, or the like. In general, any process capable of implementing the functions or steps described herein can be used to implement embodiments of the methods, systems, or computer program products (software program stored on a non-transitory computer readable medium).

Furthermore, embodiments of the disclosed methods, processes, modules, devices, systems, and computer program product may be readily implemented, fully or partially, in software using, for example, object or object-oriented software development environments that provide portable source code that can be used on a variety of computer platforms. Alternatively, embodiments of the disclosed methods, processes, modules, devices, systems, and computer program product can be implemented partially or fully in hardware using, for example, standard logic circuits or a very-large-scale integration (VLSI) design. Other hardware or software can be used to implement embodiments depending on the speed and/or efficiency requirements of the systems, the particular function, and/or particular software or hardware system, microprocessor, or microcomputer being utilized. Embodiments of the methods, processes, modules, devices, systems, and computer program product can be implemented in hardware and/or software using any known or later developed systems or structures, devices and/or software by those of ordinary skill in the applicable art from the function description provided herein and with knowledge of second harmonic generation and/or computer programming arts.

Furthermore, the foregoing descriptions apply, in some cases, to examples generated in a laboratory, but these examples can be extended to production techniques. For example, where quantities and techniques apply to the laboratory examples, they should not be understood as limiting. In addition, although specific chemicals and materials have been disclosed herein,

other chemicals and materials may also be employed according to one or more contemplated embodiments.

Features of the disclosed embodiments may be combined, rearranged, omitted, etc., within the scope of the invention to produce additional embodiments. Furthermore, certain
5 features may sometimes be used to advantage without a corresponding use of other features.

It is thus apparent that there is provided in accordance with the present disclosure, system, methods, and devices for probing the structure of molecules. Many alternatives, modifications, and variations are enabled by the present disclosure. While specific
10 embodiments have been shown and described in detail to illustrate the application of the principles of the present invention, it will be understood that the invention may be embodied otherwise without departing from such principles. Accordingly, Applicants intend to embrace all such alternatives, modifications, equivalents, and variations that are within the spirit and scope of the present invention.

CLAIMS

1. A molecule structure probe method comprising:
at a first time:

providing first and second indicator molecules bound to a double-stranded
oligonucleotide, the first and second indicator molecules being separated from each other
by a number of base pairs;

illuminating the first and second indicator molecules and the oligonucleotide with
an interrogating light; and

detecting second-harmonic generated light from the first and second indicator
molecules and the oligonucleotide and generating a first data profile based on the
detected light;

at a second time:

interacting a substance with the oligonucleotide;

after the interacting, further illuminating the first and second indicator molecules
and the oligonucleotide with the interrogating light; and

detecting second-harmonic generated light from the first and second indicator
molecules and the oligonucleotide and generating a second data profile based on the
detected light; and

comparing the first and second data profiles and determining an effect of the substance
on the oligonucleotide based on the comparison.

2. The method of claim 1, wherein the determining an effect comprises quantifying
a shape of the oligonucleotide.

3. The method of claim 1, wherein the determining an effect comprises quantifying
an amount of winding or unwinding of the oligonucleotide.

4. The method of claim 1, wherein the determining an effect comprises quantifying
an amount of bending of the oligonucleotide.

5. The method of claim 1, wherein the first and second indicator molecules are
chromophores.

6. The method of claim 1, wherein each of the first and second indicator molecules
is a planar molecule having its respective electronic transitions in a single plane that is oriented
perpendicular to a helical axis of the oligonucleotide.

7. The method of claim 1, wherein the first and second indicator molecules are
separated by at least ten base pairs.

8. The method of claim 1, wherein the first and second indicator molecules are
separated by no more than thirty base pairs.

9. The method of claim 1, wherein the first and second indicator molecules are intercalated into the oligonucleotide.

10. The method of claim 1, wherein the first and second indicator molecules comprise daunomycin.

5 11. The method of claim 1, wherein the first and second indicator molecules are intercalating nonlinear polarizable molecules.

12. The method of claim 1, wherein the substance is at least one of a drug, a protein, or a peptide.

10 13. The method of claim 1, wherein the oligonucleotide is bound to a spherical particle.

14. The method of claim 1, wherein the oligonucleotide is bound to a colloidal silica microparticle.

15. The method of claim 1, wherein the oligonucleotide is bound to a silica-amine microparticle having a diameter of 1 μ m or less.

15 16. The method of claim 1, wherein the interrogating light has a wavelength of 840nm.

17. The method of claim 1, wherein the interrogating light has a frequency of ω and the second-harmonic generated light has a frequency of 2ω .

20 18. A molecule structure probe method comprising: determining at least one of a shape, an orientation, spacing, or a relative movement of one or more target molecules or portions thereof responsively to measured second-harmonic generated light emitted from a pair of indicator molecules, which are coupled to the one or more target molecules.

25 19. The method of claim 18, wherein the emitted second-harmonic generated light is due to illumination of the indicator molecules with interrogating light, the second-harmonic generated light having a frequency twice that of the interrogating light.

20. The method of claim 19, wherein the interrogating light has a wavelength of 840nm.

21. The method of claim 18, wherein the one or more target molecules comprises a double-stranded oligonucleotide.

30 22. The method of claim 21, wherein the indicator molecules are intercalated in the oligonucleotide.

23. The method of claim 21, wherein the indicator molecules are separated from each other along the oligonucleotide by no more than thirty base pairs.

24. The method of claim 21, wherein a number of base pairs between the pair of indicator molecules defines the relative orientation of one of the indicator molecules with respect to the other.

25. The method of claim 21, wherein the indicator molecules comprise daunomycin.

26. The method of claim 18, wherein each indicator molecule is coupled to a different portion of a target molecule or a different target molecule from the other indicator molecule.

27. The method of claim 18, wherein each indicator molecule is a planar molecule having electronic transitions in a single plane.

28. The method of claim 18, wherein the one or more target molecules are tethered to one or more colloidal microparticles suspended in aqueous solution.

29. The method of claim 28, wherein each microparticle comprises a silica amine microsphere.

30. The method of claim 29, wherein each microsphere has a diameter of 1 μ m or less.

31. A method for monitoring oligonucleotide structure, the method comprising: using a pair of molecules intercalated into a double-stranded oligonucleotide to measure changes to the oligonucleotide structure based on second-harmonic generated interference from said pair of molecules.

32. The method of claim 31, wherein the intercalated molecules comprise daunomycin.

33. The method of claim 31, wherein the measured changes comprise a winding or unwinding of the oligonucleotide.

34. The method of claim 31, further comprising exposing the oligonucleotide to a drug, protein, or peptide, the measured changes being a result of said exposing.

35. The method of claim 34, wherein the exposing results in a winding, unwinding, or bending of the oligonucleotide.

36. The method of claim 31, wherein the second-harmonic generated interference is produced by illuminating the intercalated molecules with visible or infrared light.

37. The method of claim 36, wherein the illuminating light has a wavelength of 840nm.

38. A method for quantifying the shape, orientation, spacing, or relative movement between first and second target molecule portions, the target molecule portions being portions of a single target molecule or respective portions of two separate target molecules that are interacting, comprising:

providing a light source, a light detector, and a processor;

binding a first indicator molecule to the first target molecule portion;

binding a second indicator molecule to the second target molecule portion;

using the light source, generating a non-linear optical effect responsive to the relative
5 spacing and/or orientation between the first and second indicator molecules; and

using the light detector, measuring the non-linear optical effect and predicting therefrom,
information indicative of the shape, orientation, spacing, or relative movement between the first
and second target molecule portions.

39. The method of claim 38, further comprising outputting said information from
10 said processor.

40. The method of claim 38, wherein the binding comprises intercalating the first
indicator molecule to the first target molecule portion and intercalating the second indicator
molecule to the second target molecule portion.

41. The method of claim 38, wherein the non-linear light comprises second
15 harmonically generated light.

42. The method of claim 38, wherein the single target molecule or the two separate
target molecules comprise drugs.

43. A system for performing the method of any of claims 1-42.

44. A system for probing one or more target molecules having a pair of indicator
20 molecules coupled thereto, the system comprising:

an interrogating light source;

a detector configured to detect second-harmonic generated (SHG) light emitted by the
one or more target molecules and to generate a signal responsively to the detected SHG light;

a controller coupled to the light source and the detector, the controller being configured
25 to control the light source to illuminate the molecule and to receive the generated signal from the
detector, the controller being further configured to determine a relative orientation of the pair of
indicator molecules based on the generated signal.

45. The system of claim 44, wherein the interrogating light source comprises a pulsed
laser.

46. The system of claim 45, wherein the laser is a Ti:Sapphire laser.

47. The system of claim 44, wherein the interrogating light source produces visible or
infrared light.

48. The system of claim 47, wherein the interrogating light source produces light
having a wavelength of 840nm.

49. The system of claim 44, wherein the detector comprises a CCD camera.

50. The system of claim 44, wherein the detector comprises a spectrograph.

51. The system of claim 44, wherein the detector is configured to detect emitted light having a frequency twice that of light from the interrogating light source.

52. The system of claim 44, further comprising a memory module configured to store
5 the determined relative orientation of the pair of indicator molecules.

53. The system of claim 52, wherein the controller is further configured to determine a change in the one or more target molecules based on a comparison of a determined relative orientation with a previously stored relative orientation.

54. The system of claim 44, further comprising a microsphere to which the one or
10 more target molecules are tethered.

55. The system of claim 54, wherein the microsphere is a silica amine microsphere having a diameter of 1 μ m or less.

56. The system of claim 44, wherein the one or more target molecules comprises a
15 double-stranded oligonucleotide, and the controller is configured to determine an amount of winding, unwinding, or bending of the oligonucleotide responsively to the determined relative orientation.

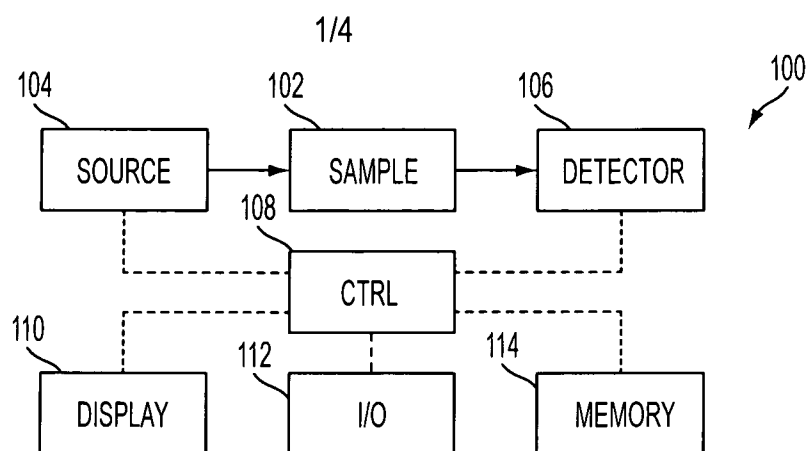


FIG. 1

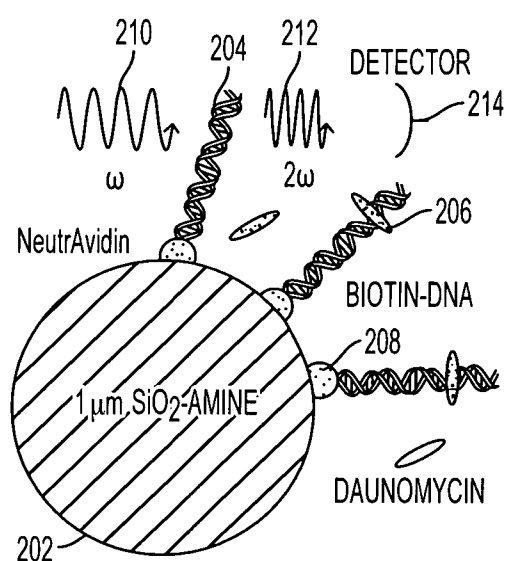


FIG. 2

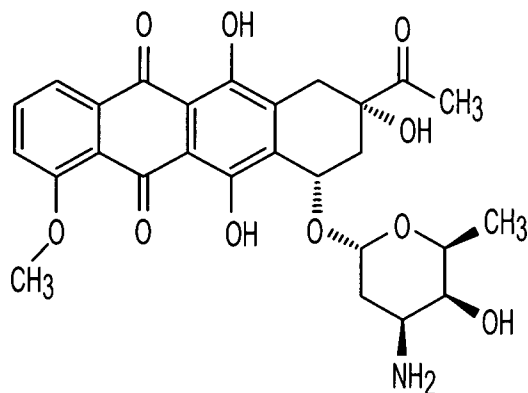


FIG. 3

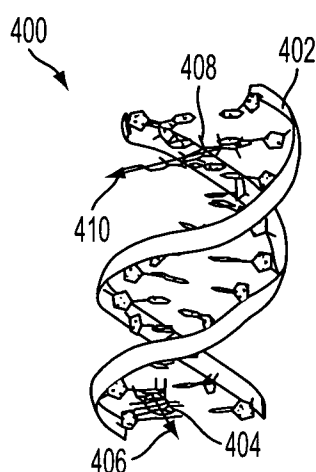


FIG. 4

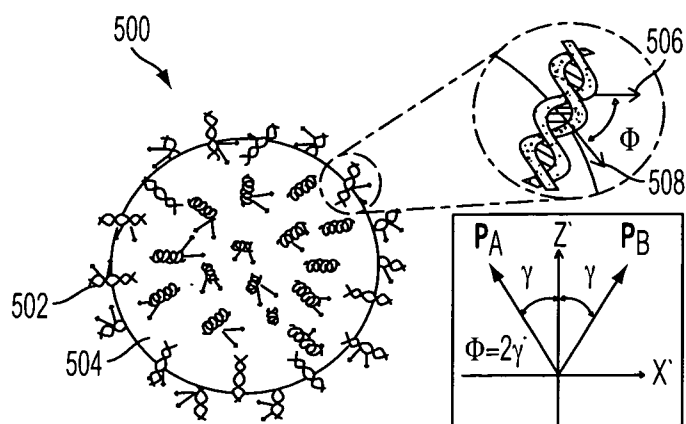


FIG. 5

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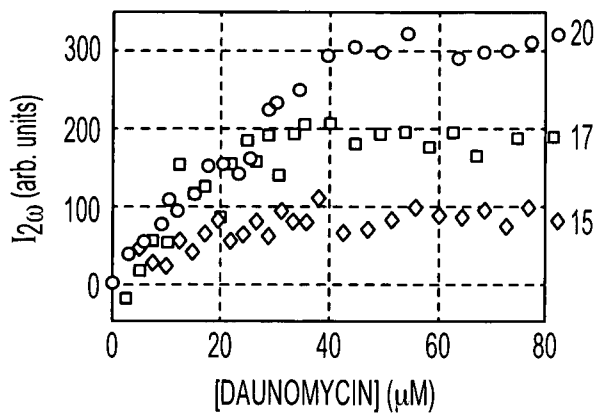


FIG. 6

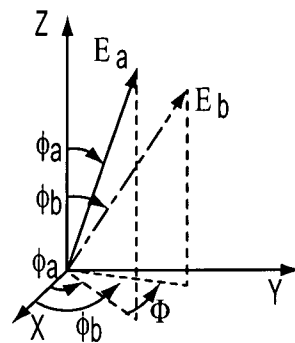


FIG. 8

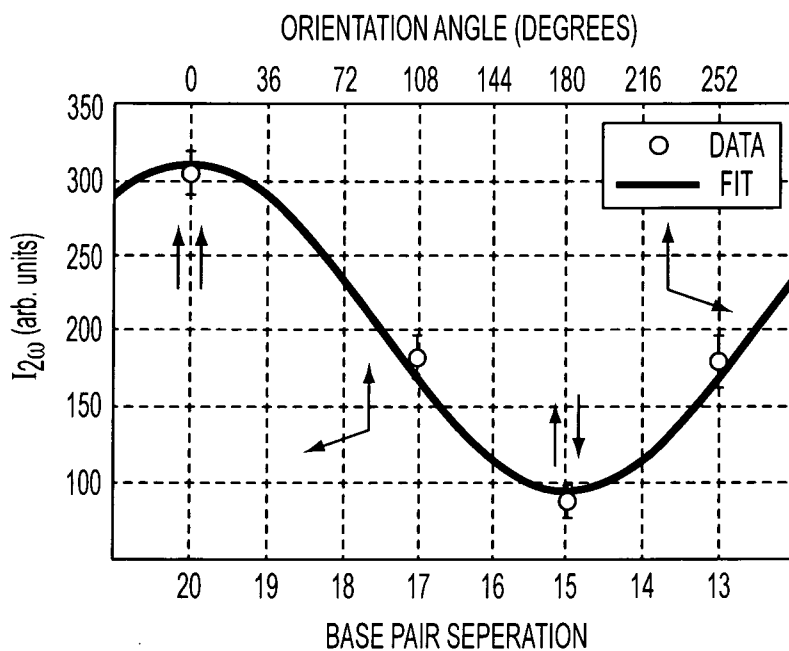


FIG. 7

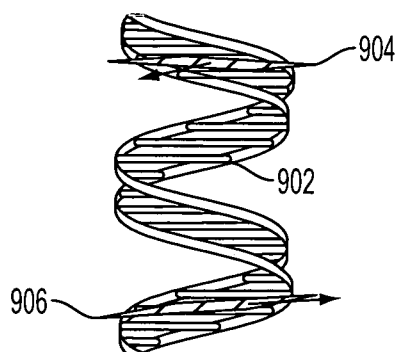


FIG. 9A

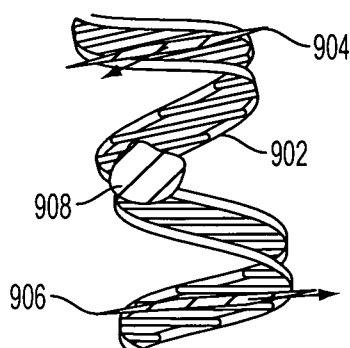


FIG. 9B

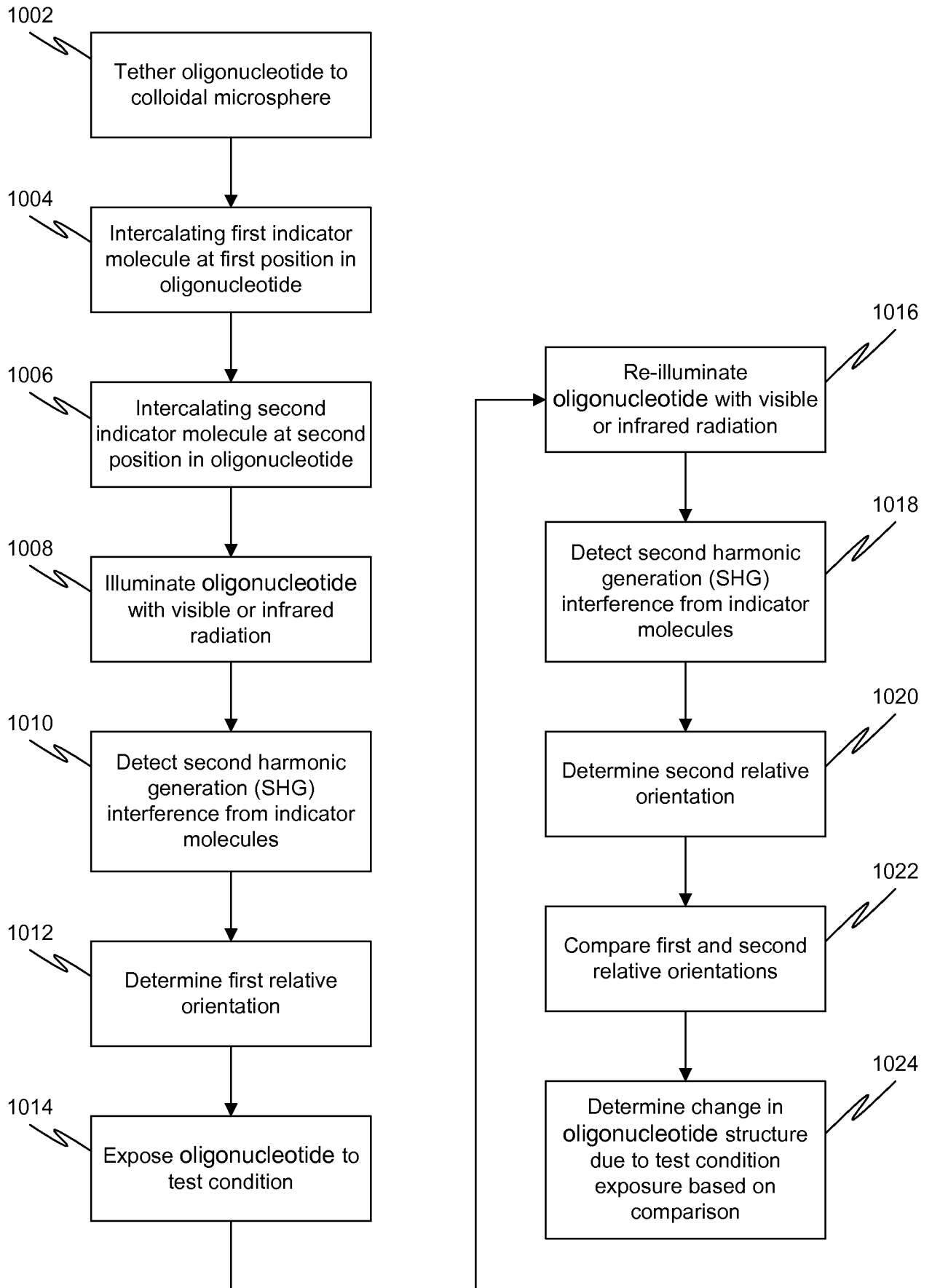
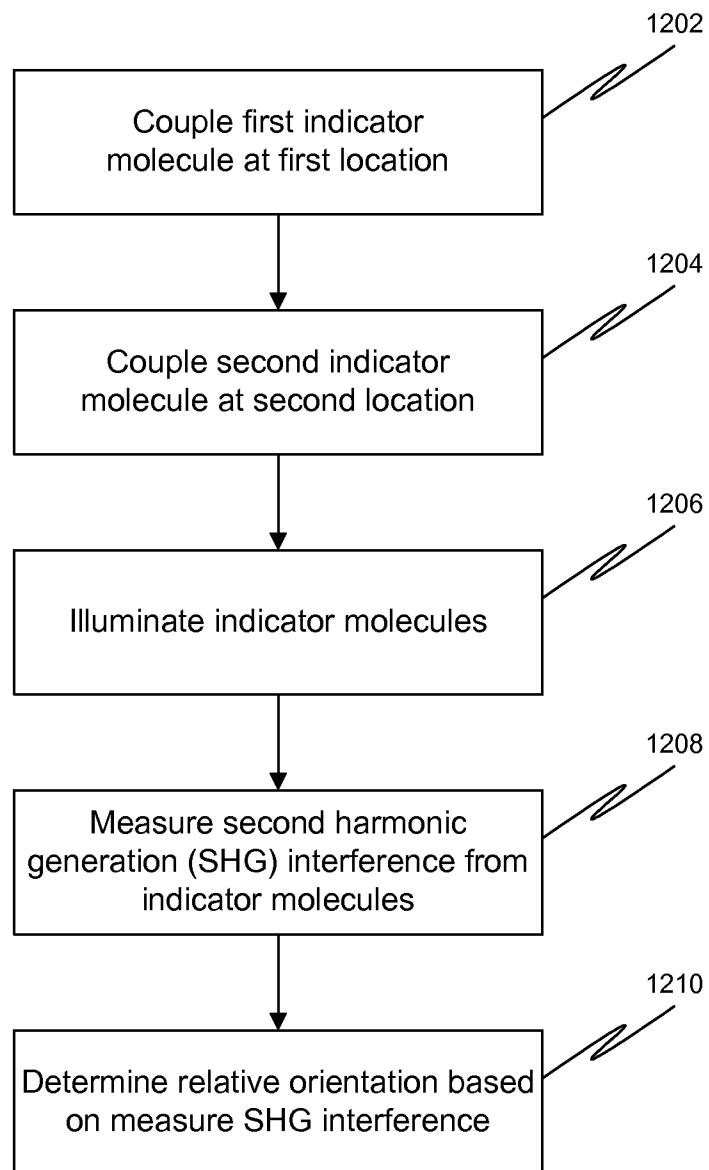
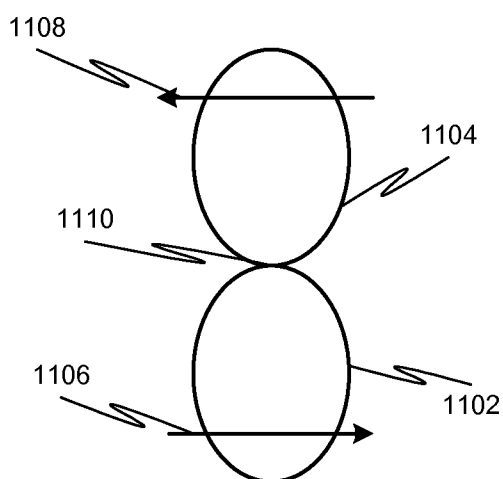
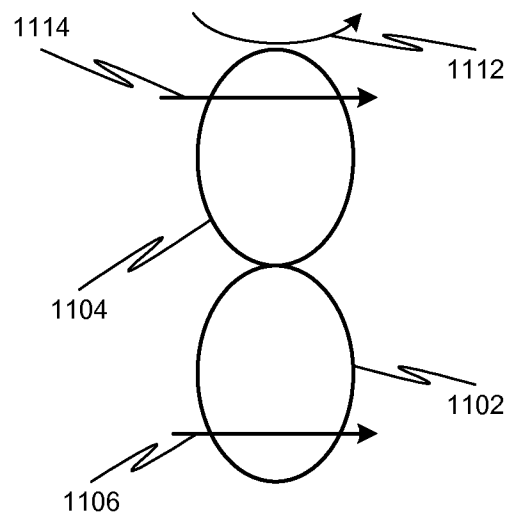


FIG. 10

**FIG. 12****FIG. 11A****FIG. 11B**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/078449

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 21/21 (2014.01)

USPC - 356/463

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)

IPC(8) - C12Q 1/68; G01N 21/00, 21/01, 21/17, 21/21, 21/39, 33/15, 33/48, 33/50, 33/53, 33/543, 33/546, 33/552 (2014.01)

USPC - 356/450, 451, 463; 435/4, 6.1, 7.92, 287.2, 288.7; 436/63, 164, 501, 518, 527, 534

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

CPC - A61K 38/00; B01J 2219/00722; C12Q 1/6883; G01B 9/02; G01C 19/72; G01J 3/453; G01N 21/6428, 31/22, 33/5094, 33/54313, 33/54366, 33/552, 33/564 (2014.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit, Google Patents, Google Scholar,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0202296 A1 (EISENTHAL) 09 August 2012 (09.08.2012) entire document	18-22, 25-28, 38-54, 56
Y		1-17, 23, 24, 29-37, 55
Y	QUIGLEY et al. "Molecular structure of an anticancer drug-DNA complex: Daunomycin plus d (CpGpTpApCpG)" Proceedings of the National Academy of Sciences, 01 December 1980 (01.12.1980), Vol. 77, No. 12, Pgs. 7204-7208. entire document	1-17, 23, 24, 29-37, 55
Y	US 2004/0067503 A1 (TAN et al) 08 April 2004 (08.04.2004) entire document	14, 15, 29, 30, 55
A	DOUGHTY et al. "Binding and cleavage of DNA with the restriction enzyme EcoR1 using time-resolved second harmonic generation" Proceedings of the National Academy of Sciences, 13 December 2011 (13.12.2011), Vol. 108, No. 50, Pgs. 19979-19984. entire document	1-56
P, A	DOUGHTY et al. "Probing the relative orientation of molecules bound to DNA through controlled interference using second-harmonic generation" Proceedings of the National Academy of Sciences, 09 April 2013 (09.04.2013), Vol. 110, Pgs. 5756-5758. entire document	1-56

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

* Special categories of cited documents:

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

27 February 2014

Date of mailing of the international search report

19 MAR 2014

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