

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
30 November 2000 (30.11.2000)

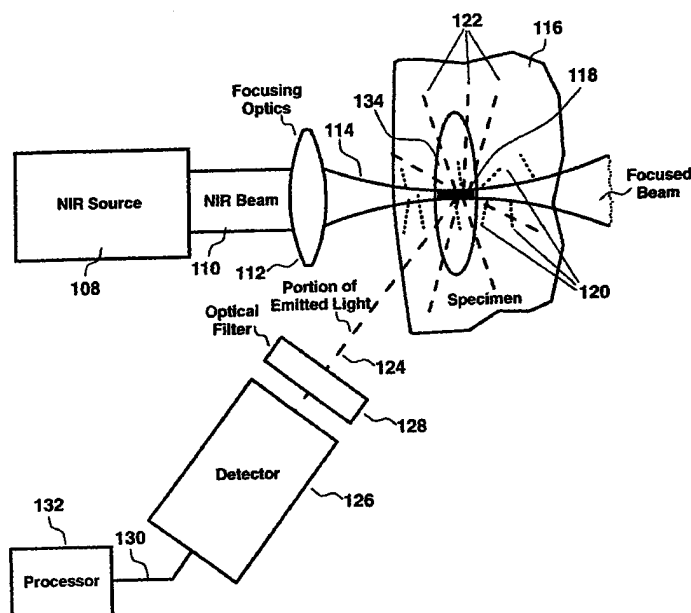
PCT

(10) International Publication Number
WO 00/71028 A1

- (51) International Patent Classification⁷: A61B 6/00 (74) Agents: MANZO, Edward et al.; Cook, Alex, McFarron, Manzo, Cummings & Mehler, Ltd., 200 W. Adams St., Ste. 2850, Chicago, IL 60606 (US).
- (21) International Application Number: PCT/US00/14169
- (22) International Filing Date: 23 May 2000 (23.05.2000) (81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/135,886 26 May 1999 (26.05.1999) US
09/569,982 10 May 2000 (10.05.2000) US
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- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— With international search report.
— Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

[Continued on next page]

(54) Title: IMPROVED METHODS AND APPARATUS FOR MULTI-PHOTON PHOTO-ACTIVATION AND DETECTION OF MOLECULAR AGENTS



(57) Abstract: A method and apparatus for diagnosis or imaging of a particular volume of material, including the steps of treating the particular volume of material with light sufficient to promote a multi-photon excitation of at least one photo-active agent contained on or within the particular volume of the material, wherein the at least one photo-active agent becomes active in the particular volume of the material, thereby resulting in the production of an optical signal, and wherein such optical signal is captured in electronic form for diagnosis or imaging of the particular volume of material.



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IMPROVED METHODS AND APPARATUS FOR MULTI-PHOTON PHOTO-ACTIVATION AND DETECTION OF MOLECULAR AGENTS

CROSS REFERENCES TO RELATED MATERIALS

5 The present application is based on provisional application no. 60/135,886 which is
a continuation-in-part of U.S. Patent Application No. 09/072,962 which is a divisional
of U.S. Patent No. 5,832,931 (based on application 741,370 filed October 30, 1996) and
a continuation-in-part of U.S. Patent Application No. 09/096,832, filed on June 12, 1998
which is a continuation-in-part of U.S. Patent No. 5,829,448 (based on application
10 739,801 filed October 30, 1996).

FIELD OF THE INVENTION

15 The present invention relates generally to methods and apparatus for achieving
selective photo-activation of one or more molecular agents with a high degree of spatial
control and for improving the detection of the diagnostic signals thereby produced. The
methods taught herein for the present invention for achieving selective photo-activation
utilize the special properties of non-linear optical excitation for promotion of an agent
from one molecular energy state to another with a high degree of spatial and molecular
specificity. The special features of the methods of the present invention are applicable
20 for activation of various endogenous and exogenous diagnostic or imaging agents, and in
particular afford distinct advantages in the diagnosis of diseases in humans and animals.
Specifically, the non-linear, multi-photon excitation methods of the present invention
facilitate controlled activation of diagnostic or imaging agents in deep tissue or in other
specimens using near infrared to infrared radiation, which is absorbed and scattered to a
25 lesser extent than during the methods and radiations currently used. By combining these
non-linear excitation methods with advanced signal encoding and processing methods,
sensitivity in the detection of diagnostic signals is greatly increased. Further, certain
aspects of the present invention have direct applicability in microscopy and other related
fields.

BACKGROUND OF THE INVENTION

30 An urgent need exists in many fields, and especially in the medical diagnostics and
imaging fields, for a method that is capable of selectively controlling the remote activation

of various molecular agents while producing few side effects. The desired improvements in activation include (1) enhancements in spatial or temporal control over the location and depth of activation, (2) reduction in undesirable activation of other co-located or proximal molecular agents or structures, (3) increased preference in the activation of desirable molecular agents over that of undesirable molecular agents, and (4) improved sensitivity in detection of the resultant diagnostic signals thereby produced. Various linear and non-linear optical methods have been developed to provide some of these improvements for some agents under very specialized conditions. However, in general, the performance and applicability of these methods have been less than desired. As a result, improved photo-activation and detection methods are needed to selectively probe a variety of molecular diagnostic agents while providing improved performance in the control of application and detection of this photo-activation.

Application of optical radiation to probe or transform molecular agents has been known for many years. For example, linear single-photon optical excitation has been used extensively for activation of molecular therapeutic agents in photodynamic therapy (PDT) and as a means for remotely interrogating diagnostic dyes and other materials. A generalized Jablonski diagram for such activation is shown in FIGURE 1(a), wherein single-photon excitation (10) occurs when a photo-active agent is excited from a lower quantum-mechanically allowed state S_m to a higher quantum-mechanically allowed state S_n upon absorption of a certain energy E_1 which is provided by interaction of a single photon P_1 with the agent. Relaxation R_1 , in the form of photochemical transformation or as radiative emission of light or other energy useful for detection or characterization of the activated molecular agent, may then occur from this transient excited state S_n . Additionally, similar relaxation processes R_2 may occur from a secondary long-lived activated state T_m , the latter occurring following intersystem crossing IX. Unfortunately, the performance of such excitation methods has not been as successful as desired. For example, the high energy optical radiation P_1 typically used can often produce disease or other undesirable side effects and may have a less than desirable penetration depth because of optical scatter and the absorbance of the UV or visible activating optical radiation.

Various multi-photon optical excitation methods have also been used in a number of laboratory applications in an effort to achieve specific improvements in the selectivity of photo-activation for certain applications, and to address many of the limitations posed by

single-photon excitation. For example, simultaneous two-photon excitation has been used as a means for stimulating fluorescence emission from molecules present in optically dense media. A generalized mechanism for such activation is shown in FIGURE 1(b), wherein simultaneous two-photon excitation (12) occurs when a photo-active agent is excited upon absorption of a certain energy E_1 that is provided by the simultaneous, combined interaction of two photons P_1' and P_2' with the agent. Note that if the energies of both photons P_1' and P_2' are identical, the excitation process is termed "degenerate". If the energies are different, the process is termed "non-degenerate". The simultaneous interaction of the two photons is frequently described as being mediated by a transient virtual state V with a lifetime on the order of 10 femtoseconds (fs) or less. Unless both photons interact with the agent during this lifetime, excitation does not occur, and the agent fails to reach state S_n . Once the agent has been promoted to the higher quantum-mechanically allowed state S_n , its photochemical and photophysical properties will be identical to those resulting from single-photon excitation (10 in Fig. 1a). Two-photon excitation has been described for use in microscopy and as an in vitro probe of membrane properties.

Far less commonly, simultaneous three-photon excitation has been used in laboratory settings to probe the spectroscopy of various molecules, including ammonia, benzene, butadiene, and nitric oxide, while simultaneous four-photon excitation has seen even more limited application in studies of molecules such as NO_2 and butadiene. The general theory of such multi-photon spectroscopies (number of photons ≤ 4) has previously been described (see, for example, Andrews and Ghoul, J. Chem. Phys. 75 (1981) 530-538). Recently, Lakowicz and co-workers have described the use of degenerate, simultaneous three-photon excitation to study the properties of various fluorophors in the condensed phase and as a possible imaging means for laser scanning microscopy (see Gryczynski et al., Photochem. Photobiol. 62 (1995) 804-808; Szmecinski et al., Biophys. J. 70 (1996) 547-555).

Additional work using multi-photon excitation has sought to elucidate the physical and chemical properties of agents using complex multi-photon excitation methods or to exert quantum control over excited-state reaction pathways using one or more temporally- or spectrally-tailored laser pulses. These reported multi-photon methods, however, generally require staged, sequential application of light energy over periods far in excess of 10 fs

in order to allow intra-molecular reorganization to occur. FIGURE 1(c) shows a generalized representation of such multi-photon excitation, wherein 3+2-photon excitation (14) occurs when a photo-active agent is initially excited to a first higher quantum-mechanically allowed state S_n upon absorption of a certain energy E_1 that is provided by the simultaneous, combined interaction of three photons P_1'' , P_2'' and P_3'' with the agent (this interaction is mediated by two virtual states, V_1'' and V_2''). Subsequent excitation occurs upon absorption of a certain additional energy E_2 that is provided by the interaction of the agent with two additional photons P_4'' and P_5'' to promote it to a second, higher state, S_p . Typically, there exists a short temporal delay between these two steps E_1 and E_2 .

Surprisingly, despite the considerable body of theoretical and experimental work with multi-photon spectroscopy and the widespread availability of ultrashort pulsed sources (i.e., laser sources capable of producing pulses of light having pulse widths generally less than approximately 10 picoseconds), the inventors of the present application are aware of no reports earlier than the present invention of the application of ultrashort pulsed, multi-photon methods for practical diagnostic imaging of human or animal tissue involving selective activation of endogenous (naturally present) or exogenous (externally supplied) molecular agents.

Denk et al. (W. Denk, J.P. Strickler and W.W. Webb, "Two-Photon Laser Microscopy," U.S. Patent No. 5,034,613) disclose the construction and use of a special epi-illumination confocal laser scanning microscope utilizing non-linear laser excitation to achieve intrinsically high three-dimensional control in the photo-activation of various exogenous agents in the laboratory on a cellular or sub-cellular scale. Emitted fluorescent light is collected by the excitation objective using an epi-illumination configuration. This light is then used for generation of luminescence-based images at the cellular and sub-cellular level for specimens mounted on a slide or other form of sample stage. In later work, Denk et al. (W. Denk, D.W. Piston and W.W. Webb, "Two-Photon Molecular Excitation in Laser-Scanning Microscopy," in Handbook of Biological Confocal Microscopy, Second Edition, J.B. Pawley, ed., Plenum Press, New York, 1995, pp. 445-458) discuss an external whole area detection method allegedly useful for collection of microscopic imaging data produced from two-photon excited fluorescent tags. This method, which the authors state as being "as yet untried," allegedly eliminates the need

to collect backscattered fluorescent light using epi-illumination. Denk points out that this approach could be useful if the microscope objective does not transmit the emitted fluorescent wavelengths, but that it is "vulnerable to contamination from ambient room light." In both of these works, no apparent method is used or anticipated for reduction of background interference from either ambient light or from scattered excitation light.

In fact, the low efficiency of non-linear excitation can translate into a very high ratio of scattered excitation light to diagnostically-useful emission. Use of various modulation methods for reduction of interference from scattered excitation light, as well as from interferences from ambient light and from other environmental and instrumental background sources, have been tried by a number of investigations. In the field of two-photon excited fluorescence, Lytle and co-workers (R.G. Freeman, D.L. Gilliland and F.E. Lytle, "Second Harmonic Detection of Sinusoidally Modulated Two-Photon Excited Fluorescence," *Analytical Chemistry*, 62 (1990) 2216-2219; and W.G. Fisher and F.E. Lytle, "Second Harmonic Detection of Spatially Filtered Two-Photon Excited Fluorescence," *Analytical Chemistry*, 65 (1993) 631-635) discuss complex methods for rejection of scattered laser excitation light by making use of second-harmonic detection methods: i.e. when sinusoidal modulation of the excitation light is performed at one frequency, and detection of the two-photon excited fluorescence is performed at twice that frequency (which is the second harmonic of the excitation modulation frequency), interferences from scattered excitation light are allegedly virtually eliminated. And by proper selection of the modulation frequency to avoid electronic and other noise frequencies, rejection of instrumental and environmental interferences is extremely high.

Hence, non-linear excitation can be used under laboratory conditions to excite various luminescent molecular agents using light at longer wavelengths than that used for linear single-photon excitation, and that the excitation thereby effected can improve three-dimensional spatial control over the location of excitation, can reduce interference from absorption and scatter of the excitation light in optically dense media, and can reduce collateral damage along the excitation path to living cell samples undergoing microscopic examination.

Thus, while work prior to that of the present inventors demonstrates many attractive features of multi-photon excitation and the use of ultrashort pulsed sources for effecting photo-activation of various materials in laboratory settings, this work has failed to achieve

selective photo-activation of one or more molecular agents with a high degree of spatial control and efficiency to meet the diverse needs of the medical field. For example, while prior work exemplified by these cited examples clearly demonstrates many attractive features of various non-linear excitation methods that are applicable for microscopic imaging uses, a general method for achieving selective photo-activation of one or more molecular agents with a high degree of spatial control that is capable of meeting the diverse needs of the medical imaging and diagnostic industry has not been previously disclosed. Specifically, there appears to be no teaching of the use of or practical methods for effecting this control on target agents and materials and on physical scales that are significant for medical and other imaging and diagnostic applications.

It is, therefore, an object of the present invention to overcome the deficiencies in the prior work.

SUMMARY OF THE INVENTION

The present invention is directed to a method and apparatus for photo-activating a molecular agent in a particular volume of material using multi-photon excitation and for improving the detection of the resultant diagnostic signals thereby produced.

More specifically, the present invention utilizes the unique physical properties of non-linear optical excitation of one or more endogenous or exogenous diagnostic or imaging agents to effect improved spatial control over the photo-activation of such agents via multi-photon excitation processes. Such multi-photon excitation results from an essentially simultaneous interaction of two or more photons with one or more endogenous or exogenous agents, wherein said photons are provided by a single ultrashort laser pulse having a duration of approximately 10 ps or less. In a preferred embodiment of the present invention, the energy and wavelength of the two or more photons are identical, and as such the excitation processes are termed degenerate. The present invention, however, is not limited to a process wherein the energy and wavelength of the two or more photons are identical.

The multi-photon excitation of the present invention allows for a number of detectable end points, including those resulting from one or more of the following events: electronic excitation of the one or more agents to a higher quantum-mechanically allowed state; vibrational excitation of the one or more agents to a higher quantum-mechanically allowed state; vibronic excitation (combined vibrational and electronic excitation) of the one or

more agents to a higher quantum-mechanically allowed state; and photoionization of the one or more agents. From such excited state end points, the one or more photo-activated agents are made to precipitate desired diagnostic effects, such as luminescence or loss of such luminescence.

5 In a preferred embodiment of the present invention, the step of photo-activation includes the step of modulating light from a light source with a particular type of modulation, thereby producing a modulated light, and treating a target subject with the resultant modulated light so as to promote multi-photon excitation of one or more endogenous or exogenous diagnostic or imaging agents present on or within the target
10 subject. It is further preferred that the present invention include the steps of demodulating a resultant detected energy signal with the particular type of modulation, and producing a demodulated energy signal which is characteristic of the particular target subject.

Even more preferably, the step of demodulating the detected energy signal with the particular type of modulation includes demodulating the detected energy signal at a
15 frequency twice or more of that of the particular type of modulation, thereby detecting the harmonic of the particular type of modulation. It is also more preferred that the demodulated energy signal which is characteristic of the particular subject represents a change in lifetime of at least one photo-activated agent present in the particular subject. The method of the present invention, however, can be performed without these optional
20 steps of modulation or demodulation.

The multi-photon excitation methods taught herein offer specific advantages relative to prior methods, including reduction of interference from absorption and scattering processes originating from the environment surrounding the excited agent, improved activation depths, improved efficiency, and enhanced control over location and specificity
25 for the excited agent.

BRIEF DESCRIPTION OF THE DRAWINGS

In describing the preferred embodiments, reference is made to the accompanying drawings:

30 FIGURES 1(a)-(c) illustrate example energy level diagrams for typical linear and non-linear optical excitation processes;

FIGURES 2(a)-(d) illustrate typical modified Jablonski energy level diagrams for several linear and non-linear optical excitation processes;

FIGURE 3 shows a comparison of two-photon excitation response and three-photon excitation response for Indo-1 as a function of excitation power;

FIGURE 4 shows a comparison of spatial excitation properties for linear and non-linear excitation processes;

FIGURES 5(a)-(b) illustrate examples of spatially localized multi-photon excitation used to locally activate agents present at the surface of tissue or below the surface of tissue;

FIGURES 6(a)-(b) show a comparison of single-photon and two-photon excited fluorescence of the dye molecule Coumarin-480 distributed evenly throughout a block of agarose gelatin;

FIGURE 7 compares absorption cross-sections as a function of excitation wavelength for Hp-IX when using single-photon excitation and simultaneous two-photon excitation;

FIGURE 8 shows example absorption and scatter spectra for human tissue covering the ultraviolet to infrared spectral region;

FIGURE 9 shows the general trends in optical absorption and scattering properties of tissue for incident short wavelength and long wavelength light;

FIGURE 10 shows a diagram of a specific preferred embodiment of the present invention for imaging endogenous or exogenous diagnostic imaging agents;

FIGURE 11 shows a diagram of an alternate preferred embodiment of the present invention for imaging endogenous or exogenous diagnostic imaging agents, wherein modulation is used to improve imaging performance; and

FIGURE 12 shows a diagram of a second alternate preferred embodiment of the present invention for videographic imaging of superficial features.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

The present invention described herein utilizes the unique physical properties of non-linear optical excitation of one or more endogenous or exogenous imaging or diagnostic agents to effect improved control over the photo-activation of such agents via multi-photon excitation processes. "Non-linear optical excitation" is defined for the purposes

of this patent document as those excitation processes involving the essentially simultaneous interaction of two or more photons with the one or more agents. “Essentially simultaneous interaction” is defined for the purposes of this patent document as those excitation processes occurring as a result of interaction of the one or more agents with photons provided by a single ultrashort laser pulse having a duration of approximately 10 ps or less. “Multi-photon photo-activation” is thus defined for the purposes of this patent document as non-linear optical excitation occurring as a result of the essentially simultaneous interaction of two or more photons originating from a single ultrashort laser pulse with one or more imaging or diagnostic agents to produce one or more photo-activated imaging or diagnostic agents. According to this definition, the physical process resulting in multi-photon photo-activation is multi-photon excitation of the one or more agents. Finally, “endogenous agents” are defined for the purposes of this patent document as photo-active materials which are pre-existent in a patient or other target, such as for example various natural chromophoric agents, such as melanin, hemoglobin, carotenes, NADH and NADPH, and other photo-active materials, such as tattoo dyes. Conversely, “exogenous agents” are defined as photo-active materials which are not pre-existent in a patient or other target, such as for example various diagnostic dyes or other photo-active agents administered for the purpose of increasing efficiency of conversion of optical energy into a diagnostic or therapeutic signal.

The end point of such multi-photon photo-activation according to the present invention may include one or more of the following detectable events: electronic excitation of the one or more agents to a higher quantum-mechanically allowed state; vibrational excitation of the one or more agents to a higher quantum-mechanically allowed state; vibronic excitation (combined vibrational and electronic excitation) of the one or more agents to a higher quantum-mechanically allowed state; and photoionization of the one or more agents. From such excited state end points, the one or more photo-activated agents are made to precipitate desired diagnostic effects, such as luminescence or loss of such luminescence.

Multi-photon excitation according to the present invention is performed at a wavelength approximately twice or more of that used for conventional single-photon excitation. By focusing a beam of this optical radiation at or into a specimen under examination, one or more diagnostic agents may be photo-activated at a location

substantially limited to a focal region defined by the focused beam, resulting in unprecedented resolution along and perpendicular to the optical path.

Since photo-activation according to the present invention is performed at long wavelengths relative to corresponding linear excitation processes, scatter and absorption of the excitation energy are greatly reduced. For thick, optically-dense samples, such as human tissue, this means that multi-photon excitation is possible at depths considerably greater than those possible using conventional linear excitation methods. Moreover, it is not necessary for the light emitted from the diagnostic agent or agents to be detected or imaged directly without scatter, since spatial information concerning the origin of the emitted light is encoded by and may be correlated to the focus of the activating beam. Thus, by moving the location of this focus relative to the specimen, either by moving the light source or the target, or both, or otherwise varying the optical path, a two- or three-dimensional image of the resultant emitted energy signal may be developed. Also, by modulating the activation light and using an appropriate demodulation method on the detection apparatus, rejection of scattered excitation light and other interferences may be markedly improved.

The non-linear optical excitation of the present invention has additional advantages during photo-activation of imaging or diagnostic and other agents, including reduction of collateral excitation and damage along the excitation path, reduction in exposure to harmful optical wavelengths, and enhanced specificity in the excitation of the agent. The non-linear optical excitation approach employed in the present invention provides a superior means for the detection and imaging of many diseases.

The present invention is intended primarily for in vivo detection and imaging of disease and other characteristics of tissues, such as cancer in the human breast. However, it will be clear once the invention is fully disclosed that the methods and apparatus taught have numerous additional applications, and that these methods and apparatus can be applied to other fields, such as laser scanning microscopy, in order to achieve substantive improvements in the performance characteristics of methods and apparatus used in such fields.

The basic configurations of the multi-photon photo-activation method and apparatus for achieving such imaging or diagnostic outcomes are described in U.S. Patent Application Serial nos. 08/741,370 and 09/096,832, which are assigned to the assignee

of the present invention and which have inventors in common with the present application. U.S.S.N. 08/741,370 filed October 30, 1996 and U.S.S.N. 09/096,832 filed on June 12, 1998, are incorporated herein by reference in their entirety.

In brief, embodiments of the present invention include a light source, details of which are described in detail below, where the resulting light is directed at a tissue or substance to be imaged. A modulation device or method can be included, as also described. The region to be imaged can be moved relative to the light beam or source or alternatively, the light beam can be moved. A detector is provided, and may include a suitable demodulator if modulation has been used.

Details of the light used for photoactivation will now be set forth through a discussion of the scientific principles used in this invention.

Energy level models of multi-photon photo-activation:

One aspect of the present invention taught in this disclosure lies in the use of multi-photon processes to selectively and efficiently photo-activate one or more imaging or diagnostic agents with a high degree of spatial control. This selective photo-activation is achieved by means of harnessing the special properties of non-linear optical excitation to promote an agent from one molecular energy state to another. To fully understand the salient features of this process, a conceptual model of multi-photon excitation is developed herein. This is conveniently achieved through use of energy level diagrams for representative cases. FIGURES 2(a)-(d) illustrate typical modified Jablonski energy level diagrams for several linear and non-linear optical excitation processes.

FIGURE 2(a) illustrates single-photon excitation which occurs when an agent is excited from an initial quantum-mechanically allowed state S_i (which is typically the ground state) to a final quantum-mechanically allowed state S_f upon absorption of a certain energy E_i that is provided by interaction of a single photon P with the agent. A number of different allowed final states are possible. Each allowed state may be further subdivided into an ensemble of discrete sub-states, such as various vibrational states superimposed on a particular electronic state. Hence, each allowed state S_i and S_f may constitute a complex band of allowed states that reflect the fundamental properties of the agent and its local environment. In the case of a luminescent imaging agent, promotion of the agent from S_i to S_f can initiate various radiative emissions of light from the excited

state, including for example fluorescent or phosphorescent emission of light. As an example, the dye molecule coumarin-480 may, upon absorption of a single photon of light at 400 nm, emit a photon via fluorescence re-emission (which may also be described as a relaxation process) at 465 nm. In single-photon excitation (20), the probability of excitation is linearly related to the irradiance of the incident optical radiation, so single-photon excitation (20) is referred to as a linear excitation process.

In FIGURE 2(b), simultaneous two-photon excitation (22) occurs when a photo-active agent is excited from an initial quantum-mechanically allowed state S_i to a final quantum-mechanically allowed state S_f upon absorption of a certain energy E_i that is provided by simultaneous interaction of two photons P_1 and P_2 with the agent.

For all examples consider henceforth, it will be assumed for illustrative purposes that the energy and wavelength of the two (or more) photons involved in excitation are identical, and as such that the excitation process is termed "degenerate." The present invention, however, is not limited to degenerate processes but is also applicative to when the energy and wavelength of the two (or more) photons are not identical.

Once the agent has been promoted to the final quantum-mechanically allowed state S_f its photochemical properties will be identical to those resulting from single-photon excitation (20).

The simultaneous interaction of the two photons illustrated in Fig. 2(b) is frequently described as being mediated by a transient virtual state V_i with a lifetime on the order of 10 fs or less. If both photons do not interact with the agent during the life time of this virtual state, excitation does not occur, and the agent returns to allowed state S_i . Due to the exceedingly short lifetime of the virtual state V_i , the instantaneous irradiance, or $W \text{ m}^{-2}$ of the incident excitation light must be sufficiently high to yield significant efficiency in absorption of the second photon P_2 before the virtual energy state V_i undergoes relaxation back to S_i . Hence, pulsed excitation sources having very high peak powers are commonly used to efficiently stimulate this process; such sources are often preferable since they are capable of providing large numbers of photons to the excited agent during the brief lifetime of the virtual state V_i .

An example of simultaneous two-photon excitation (22) is the promotion of fluorescence emission of light at 465 nm from coumarin-480 upon simultaneous absorption of two photons at 800 nm. In this example, the probability of excitation is

related to the product of the instantaneous irradiance of the first photon P_1 and the second photon P_2 . This can be conceptualized in the form of a photochemical reaction,



which shows that an agent in the ground state is promoted to an excited state following simultaneous absorption of two photons, $h\nu_{800 \text{ nm}}$, each at 800 nm. The rate R describing production of the excited state is given by $R = k [\text{Agent}_{\text{GROUND STATE}}] [h\nu_{800 \text{ nm}}]^2$, where k is an intrinsic constant for the agent, such as absorbance cross-section, and where $[\text{Agent}_{\text{GROUND STATE}}]$ and $[h\nu_{800 \text{ nm}}]$ symbolize concentrations of agent molecules in the ground state and the excitation photons, respectively. Hence, due to the quadratic dependence of the rate expression on instantaneous irradiance, simultaneous two-photon excitation (22) is referred to as a non-linear excitation process.

In FIGURE 2(c), simultaneous three-photon excitation (24) occurs when a photo-active agent is excited from an initial quantum-mechanically allowed state S_i to a final quantum-mechanically allowed state S_f upon absorption of a certain energy E_i that is provided by simultaneous interaction of three photons P_1' , P_2' and P_3' with the agent. The simultaneous interaction of the three photons is described as being mediated by two transient virtual states V_1' and V_2' each having a lifetime on the order of 10 fs or less. If all three photons do not interact with the agent during these life times, excitation does not occur and the agent returns to S_i . Due to the exceedingly short lifetime of the virtual states V_1' and V_2' , the instantaneous irradiance of the incident excitation light must be sufficiently high to yield significant efficiency in absorption of the second photon P_2' and the third photon P_3' before the agent undergoes relaxation back to S_i .

An example of simultaneous three-photon excitation (24) is the promotion of fluorescence emission of light at 465 nm from coumarin-480 upon simultaneous absorption of three photons at 1200 nm. In this example, the probability of excitation is related to the product of the instantaneous irradiance of the first photon P_1' , the second photon P_2' and the third photon P_3' . This can be conceptualized in the form of a photochemical reaction,



which shows that an agent in the ground state is promoted to an excited state following simultaneous absorption of three photons, $h\nu_{1200\text{ nm}}$, each at 1200 nm. The rate R is given by $R = k [\text{Agent}_{\text{GROUND STATE}}] [h\nu_{1200\text{ nm}}]^3$, where $[h\nu_{1200\text{ nm}}]$ symbolizes the concentration of excitation photons. Hence, due to the cubic dependence on instantaneous irradiance, simultaneous three-photon excitation (24) is also referred to as a non-linear excitation process.

In FIGURE 2(d), simultaneous multi-photon excitation (26) occurs when a photo-active agent is excited from an initial quantum-mechanically allowed state S_i to a final quantum-mechanically allowed state S_f upon absorption of a certain energy E_i that is provided by simultaneous interaction of two or more photons with the agent. Once the agent has been promoted to the final quantum-mechanically allowed state S_f its photochemical properties will be identical to those resulting from single-photon excitation (20). The simultaneous interaction of the two or more photons is often described as being mediated by a corresponding multiplicity of one or more virtual states, each having a lifetime on the order of 10 fs or less. If the necessary photons do not interact with the agent during these lifetimes, excitation to S_f does not occur and the agent returns to S_i . Due to the exceedingly short lifetime of the one or more virtual states, the instantaneous irradiance of the incident excitation light must be sufficiently high to yield significant efficiency in absorption of the necessary photons before the agent undergoes relaxation back to S_i .

An example of simultaneous multi-photon excitation (26) is the promotion of fluorescence emission of light at 465 nm from coumarin-480 upon simultaneous absorption of n photons at a wavelength (λ) of $(400 \cdot n)$ nm (for example, if $n = 2$, $\lambda = 800$ nm; if $n = 3$, $\lambda = 1200$ nm; and so on for $n \geq 2$). In this example, the probability of excitation is related to the product of the instantaneous irradiance of the n photons. This can be conceptualized in the form of a general photochemical reaction,



which shows that an agent in the ground state is promoted to an excited state following simultaneous absorption of n photons. The rate R is given by $R = k [\text{Agent}_{\text{GROUND STATE}}] [h\nu]^n$, where $[h\nu]$ symbolizes the concentration of the n

excitation photons. Hence, due to the non-linear dependence on instantaneous irradiance, simultaneous multi-photon excitation (26) is also referred to as a non-linear excitation process.

5 A more general definition of multi-photon excitation than that given above requires only that the two or more photons interact with the one or more agents in a substantially simultaneous manner, for example any interaction occurring during a single ultrashort laser pulse having a duration of approximately 10 ps or less. Under such constraints, the interaction of light with the one or more agents must occur in a fast regime, substantially limiting (and localizing) the direct photo-activation effect to intra-molecular processes, such as electronic excitation or photoionization of the agent. No significant elapse of time
10 nor substantial molecular reorganization nor motion will occur during such excitation, and on conventionally observable frames of reference any transitions thereby effected in the one or more agents will occur as an essentially single, concerted step. This more general definition shall be used in subsequent descriptions of the properties of multi-photon excitation and of the resultant multi-photon photo-activation of imaging or diagnostic
15 agents.

In addition to the specific examples of photochemical processes and energy diagrams shown in reference to FIGURE 2, many other possible transitions and energy level conditions are possible, depending upon numerous factors, including the characteristics
20 of the molecular system, its environment, and the particular energies of the absorbed and released forms of energy, along with their temporal and spatial correlations.

Non-linear relationships in multi-photon photo-activation:

For the foregoing examples, once the agent has been promoted to the final quantum-mechanically allowed state S_f following absorption of a particular quantity of energy E_i , its photochemical properties, including diagnostically useful luminescent emission of light, will be identical regardless of the way used for such promotion. These properties will be determined by the intrinsic properties of the excited agent and its local environment. The particular final energy state S_f attained will be determined primarily by the magnitude of the total energy E_i delivered to the molecule, and it is thus the magnitude of E_i that will ultimately determine the properties of the agent upon arrival at S_f . Notably, when ultrashort pulsed excitation methods are used, the mechanism responsible for promoting the agent to the excited state has no significant impact on this fate since the excitation process itself does not directly impact the subsequent properties of the excited agent or its environment.

The feasibility of multi-photon excitation for a given agent can be readily evaluated through examination of agent response (such as luminescence) as a function of excitation power (which correlates directly with instantaneous irradiance). By measuring such agent response at several excitation powers, successful multi-photon excitation can be confirmed by plotting $\log_{10}(\text{response})$ against $\log_{10}(\text{power})$. This should produce a straight line with a slope of n , where n is the number of excitation photons absorbed by the agent under the particular test conditions. This is readily demonstrated for the fluorescent probe agent Indo-1, which exhibits strong single-photon excitation (emitting fluorescence at 500 nm) upon illumination with light between 300 and 400 nm. When this agent is illuminated using focused light at 810 nm and $\log_{10}$ (fluorescence signal) is plotted against $\log_{10}(\text{average power})$, as shown in FIGURE 3, a two-photon excitation response is noted (slope $m = 1.96$, correlation coefficient $R^2 = 0.99994$). The focused light is, for example, a beam composed of a 76 MHz repetition rate train of approximately 200 fs pulses produced by a mode-locked titanium:sapphire laser. When a similar test is performed at 910 nm, Indo-1 exhibits a three-photon excitation response as a function of laser power ($m = 2.97$, $R^2 = 0.991$). In comparison, the fluorescent agent Coumarin-540A, which exhibits only a two-photon response at these wavelengths, exhibits slopes of 2.01 and 2.00 at 810 nm and 910 nm, respectively. At these wavelengths, these multi-photon signals completely disappear if the laser is not pulsed,

confirming that multi-photon excitation is responsible for the observed response (since the non-pulsed laser beam does not provide sufficient instantaneous irradiance to support a multi-photon process with these agents at these wavelengths).

5 Additionally, the excitation cross-sections for multi-photon processes generally decrease as the number of photons required for a given transition increases. For example, the three-photon cross-section for a particular transition in a given agent will generally be lower than the respective two-photon cross-section for the same transition. This is at least in part due to the reduced probability that all photons necessary for a particular multi-photon process will interact with the agent in a substantially simultaneous manner. This is evident when comparing the magnitude of the two-photon excitation response (30) and the three-photon excitation response (32) for Indo-1 (FIGURE 3) at a given average excitation power. However, since the slope of the three-photon process is steeper than that of the two-photon process, this difference in relative cross-section can be substantially ameliorated by increasing the instantaneous irradiance of the excitation light, for example
10 by decreasing pulse width for a beam of a given average power, or by increasing pulse energy (for example by using an amplified light source, such as a regeneratively amplified titanium:sapphire laser or a chirp-pulse amplified Nd:YAG laser).
15

Spatial properties of multi-photon photo-activation:

Because multi-photon excitation is non-linear with instantaneous irradiance, such processes exhibit an important and dramatic difference in spatial excitation properties relative to linear excitation processes. For example, as shown in FIGURE 4, if a laser beam is focused into a material, a beam intensity profile (40) will be produced that varies as a function of distance through the sample, reaching a maximum level at the center of the focus, as predicted by classical Gaussian optical theory. For a single-photon process, the linear relationship between beam intensity (or instantaneous irradiance) and excitation efficiency results in a single-photon excitation efficiency profile (42) that follows the beam intensity profile (40). In contrast, for multi-photon processes, the non-linear relationship between beam intensity (or instantaneous irradiance) and excitation efficiency (such as proportionality to I^2 or I^3 for two- or three-photon processes, respectively, where I is instantaneous irradiance at any position in the sample) results in an excitation efficiency profile (44) that is significantly sharper than the beam intensity profile (40). This spatial localization of excitation can thus be used to substantially limit the extent of excitation to a small focal zone when multi-photon excitation is employed. In contrast, when linear excitation is employed, excitation occurs substantially along the entire optical path, making spatial localization of excitation considerably less defined. Such spatial localization may be observed in both transparent media, such as the eye, and in optically dense media, such as dermal tissue. Thus, spatially localized multi-photon excitation may be used to locally activate agents present at the surface of tissue (50) or below the surface of tissue (52), as shown for example in FIGURES 5(a) and 5(b), respectively.

Stimulation of a localized, remote photo-activated response in an optically dense medium is demonstrated in FIGURES 6(a) and 6(b). FIGURE 6(a) shows a photograph of a solid block of agarose gelatin (60) throughout which the dye molecule Coumarin-480 has been evenly distributed. FIGURE 6(b) is a drawing of the photograph in FIGURE 6(a). The agarose gelatin (60) constitutes an optically dense medium since it strongly scatters visible light. Coumarin-480 emits blue fluorescent light when excited via single-photon methods at 350-450 nm or when excited via two-photon methods at 700-900 nm.

In this example, a beam of light (62) was focused into the agarose gelatin (60) from one side such that it formed a focus (64) approximately 2 cm into the gelatin. Specifically,

either a beam (62) of visible light (at 400 nm) or of NIR light (at 730 nm from a mode-locked titanium:sapphire laser) was expanded to produce a collimated beam approximately 50 mm in diameter using a beam expanding telescope. The laser produced a continuous train of <200 fs pulses of light at a 76 MHz pulse repetition frequency. The expanded beam was then focused into the agarose gelatin (60) using a 250 mm focal length (f.l.), 50 mm diameter biconvex singlet glass lens. The agarose gelatin (60) was then positioned such that the focus (64) of this 250-mm f.l. lens fell at a position 2 cm into the agarose gelatin (60). Now, from a perspective looking directly down onto the agarose gelatin (60) from above the optical axis defined by the beam of light (62), FIGURES 6(a) and 6(b) clearly show that fluorescence from the Coumarin-480 is stimulated only at the focus of the NIR beam (66), while fluorescence is emitted along the entire line of flight for the visible beam (68). Moreover, a large fraction of the NIR beam (70) continued through the entire thickness of the agarose gelatin (60) with only slight attenuation, while the visible beam (72) was completely extinguished before traversing one third of the thickness of the agarose gelatin (60).

The features observed in FIGURES 6(a) and 6(b) dramatically illustrate the unique spatial localization achievable with multi-photon excitation, along with the generally improved penetration of NIR light relative to shorter wavelength light in optically dense media (such as an agarose gelatin (60) or human or animal tissue). Because of the non-linear relationship between efficiency of multi-photon excitation and instantaneous irradiance, agent stimulation at positions along the beam path prior to and after the focus is negligible. Hence, little or no photo-activation occurs outside the focal zone. Also, because the NIR excitation light is only weakly absorbed or scattered by the gelatin, sharp focus is maintained at deep penetration depths into the block (in fact, by moving the gelatin along the optical axis, sharp focus was achievable through the entire 8-cm thickness of gelatin). Since the sharpness of the focus observed in FIGURES 6(a) and 6(b) is determined by Gaussian optical properties, the length and area of the focal zone is easily adjusted by changing the optical parameters used for beam manipulation, such as beam expansion and subsequent refocusing (thereby controlling numeric aperture of the resultant focused beam).

As the number of photons employed for excitation is increased, the multi-photon excitation efficiency profile (44) shown in FIGURE 4 and evidenced in FIGURES 6(a)

and 6(b) will become increasingly tighter. Hence, three-photon excitation will provide tighter spatial localization than that possible with two-photon excitation, and so on. This implies that the multi-photon order (number of photons utilized for a particular excitation) can be optimized to match the spatial properties of the resultant focal zone to that of the target. For example, the thickness of the focal zone might be tightened through the use of three-photon excitation in order to optimize selective activation of agent at the surface of tissue (50), as shown in FIGURE 5(a).

Spectral response properties of multi-photon photo-activation:

In addition to dramatic differences in spatial excitation properties, the selection rules governing efficiency of excitation as a function of wavelength may be vastly different for single-photon and multi-photon excitation. However, the properties of the specific excited state accessed will be the same regardless of the excitation mechanism used to promote the agent to that particular excited state.

FIGURE 7 provides an example showing a relative comparison of absorption cross-sections as a function of excitation wavelength for the weakly luminescent molecule Hp-IX in ethanol when using conventional single-photon excitation (74) and simultaneous two-photon excitation (76). Additionally, the simultaneous two-photon excitation (76) data is also plotted using a wavelength scale that has been divided by two (78) to reflect that simultaneous two-photon excitation is equivalent in energy to absorption of a single photon at twice the energy (or one half the wavelength) of the each of two photons. Comparison of the relative cross-sections for single-photon excitation (74) and for simultaneous two-photon excitation plotted at the one-half wavelength scale (78) shows significant differences as a function of wavelength. Specifically, the prominent Sorrett band (80) evident with single-photon excitation (74) is absent for simultaneous two-photon excitation (76) or (78), since it is quantum-mechanically disallowed.

Such differences are attributable to differences in selection rules for particular molecular transitions that are dependent on the mechanism employed in excitation, and they are useful for optimizing efficiency or selectivity of excitation based on the differences in multi-photon selection rules and in designing specific agents with special multi-photon properties. In general, for centrosymmetric agents (those containing a center of inversion), an excitation process employing an even number of photons (such

as for example two-photon excitation) must raise an agent from its initial state to an excited electronic state having like parity. This is the exact opposite of the selection rules for an excitation process employing an odd number of photons (such as single-photon excitation), and accounts for the differences in the Sorrett band (80) response for Hp-IX observed in FIGURE 7. In contrast, agents with little or no symmetry will in general have identical selection rules regardless of the number of photons employed in their excitation. Thus, for centrosymmetric agents, it will generally be advantageous to carefully determine multi-photon spectral response as a function of wavelength in order to determine optimal excitation wavelengths, while for non-centrosymmetric agents, single-photon excitation spectra may generally be used to estimate multi-photon spectral response as a function of wavelength.

Significance of tissue absorbance and scattering properties in multi-photon photo-activation:

While the cross-section for multi-photon excitation may be considerably lower than that observed with single-photon excitation, use of the multi-photon methods may be favorable to conventional excitation methods under many conditions because of lower matrix absorption and optical scattering of longer wavelength optical radiation. For example, FIGURE 8 shows typical absorption and scattering properties for tissue covering the UV to IR spectral region. Several conclusions are clear from FIGURE 8.

First, the use of longer wavelength light generally reduces the relative effects of scatter, and thereby improves optical penetration depth. Hence, substitution of a multi-photon method according to embodiments of the present invention for a conventional, single-photon method will reduce the effects of scatter on the delivery of activating light to an agent present within tissue. For example, controlling the excitation to use two photons at 700 nm or three photons at 1050 nm to activate a UV-active fluorophor, such as Indo-1 (which is normally activated using 350 nm light) will result in an approximate 100-fold to 2000-fold reduction in scatter for 700 nm and 1050 nm light, respectively. Needless to say, this represents an enormous improvement over prior art technologies.

Second, interference from tissue absorbance, which can reduce the penetration of activating light and which might lead to light-induced tissue damage, is generally reduced through the use of longer wavelength light, for example, NIR light in the so-called tissue

transmission window extending from approximately 700 nm to 1300 nm. Negligible effects are generally observed in tissues irradiated with lower-energy, NIR light, even when the optical power of the NIR light is many-fold higher than that of the UV or visible radiation. Hence, substitution of a multi-photon method in accordance with the present invention for a conventional, single-photon method will usually reduce the effects of tissue absorbance on the delivery of activating light to an agent present within tissue. For example, use of two-photon excitation at 700 nm or three-photon excitation at 1050 nm to activate a UV-active PDT fluorophor, such as Indo-1, that is normally activated using 350 nm light, will result in an approximate 260-fold to 3500-fold reduction in absorbance for 700 nm and 1050 nm light, respectively, in normal human tissue. Hence, the use of agents that are characterized by single-photon excitation wavelengths that overlap with spectral regions of high tissue absorbance becomes feasible for deep-tissue applications through the use of multi-photon methods, since such methods enable delivery of activating light to deep tissue locations without interference from tissue absorption.

Reduced scatter and absorption by tissue allows spatially-localized excitation to be efficiently realized with multi-photon methods, as illustrated in FIGURES 5 and 6, since matrix interference is substantially reduced. Furthermore, reduced scatter and absorption by tissue results in additional safety advantages for multi-photon excitation. For example, when UV light impinges on human tissue, the majority of the optical energy is immediately absorbed and scattered in the outermost layers, such as the epidermis and dermis. This absorption may occur due to excitation of certain molecules in the cells of this tissue, such as those composing the genetic material in the cellular nucleus. This absorption of high-energy light by cellular constituents can thereby initiate a variety of collateral photochemical changes in these cells, including irreversible genetic damage and induction of cancer. In contrast, NIR light used for two-photon or three-photon methods will not be appreciably absorbed or scattered by tissue, and as such the possibility for collateral damage to cells will be substantially lower.

Additional effects of absorption and scatter are considered in FIGURE 9. The inherently high absorption and scatter of higher-energy UV or visible photons by tissue can result in very shallow tissue penetration depths, while lower-energy NIR photons generally have much greater penetration depths. Further, since scattered higher-energy photons can induce emission from diagnostic agents along their scatter path, higher-

energy photons that manage to penetrate tissue will tend to produce a diffuse emission zone that extends radially from the excitation path. However, because of the non-linear dependence on multi-photon excitation, irradiation with lower-energy photons will produce a more sharply defined excitation pattern that is not significantly blurred by the presence of scattered lower-energy photons (since such scattered photons in general cannot induce emission due to loss of spatial and temporal coherence). Hence, illumination and subsequent detection of subsurface features is difficult or impossible when using higher-energy photons 90, such as those in the UV or visible spectral regions, while illumination and subsequent detection of subsurface features is much easier when using lower-energy photons 92, such as those in the NIR or IR spectral regions. Note also that the emitted light from the diagnostic agent may be highly absorbed and scattered by the tissue or other optically dense medium under examination. However, for satisfactory detection of the emitted light, it is only necessary that a small fraction of this light make its way to a detector. The large extent to which this emitted light may be scattered implies that sophisticated methods are needed to differentiate emitted light produced by an excited agent from scattered light and other optical or instrumental noise sources. This latter consideration is the topic of a subsequent section.

These important differences in absorption and penetration depth properties for higher-energy and lower-energy light are shown schematically in FIGURE 9. When UV or visible light 90, for example light at 400 nm, impinges on human tissue 96, the majority of the optical energy is immediately absorbed 98 and scattered 94 in the outermost layers 100, such as the epidermis and dermis. Hence, optical penetration depth is low, and potential for induction of collateral photo-induced damage is high for excitation with UV or visible light 90, such as that conventionally used for linear excitation of diagnostic agents. In contrast, NIR light 92, for example at 800 nm, will experience much lower absorption and scatter by tissue 96. The overall depth of penetration will be much greater and the extent of collateral damage to cells will be substantially lower. Hence, if long-wavelength excitation light is used in a multi-photon excitation process to replace higher-energy, single-photon excitation, it becomes possible to photo-activate specific diagnostic agents present in deep tissues using relatively non-damaging wavelengths that have high penetration depths.

Selection of excitation wavelengths for multi-photon photo-activation:

The foregoing discussion on the role tissue properties play in the overall efficiency of multi-photon excitation suggests an equally important point: the order of a multi-photon process (i.e. the number of photons utilized for excitation) can be chosen to simultaneously optimize the excitation wavelength of the one or more desired photo-active agents and the transmissive properties of tissue. Specifically, to activate one or more exogenous photo-active agents, it will in general be desirable to choose a particular multi-photon process that permits excitation using light in a transmissive region for the matrix (such as tissue) and that is efficient in photo-activation of the desired exogenous agent or agents. Similarly, to activate one or more endogenous photo-active agents, it will in general be desirable to choose a particular multi-photon process that allows excitation using light in a transmissive region for the matrix (such as tissue) and that is efficient in photo-activation of the desired endogenous agent or agents. In the case where the one or more endogenous agents constitutes a major component of the tissue to be examined, proper selection of the multi-photon process will generally allow spatially-localized photo-activation of such agents even at subsurface locations (since interference from direct, linear absorption of the activating light can be minimized). This is clearly illustrated in FIGURES 6(a) and 6(b), where selective, efficient activation of an agent (66) is demonstrated deep within a specimen through which the agent is uniformly distributed. In contrast, conventional activation methods yield agent activation (68) that is not spatially localized and that exhibits poor efficiency at such depths due to absorbance of the activating light by the agent along the optical path.

Hence, an example of the selection of multi-photon process order for simultaneous optimization of photo-activation efficiency and tissue transmissive properties is the activation of Indo-1 in normal human tissue. In this example, the large two-photon cross-section of Indo-1 relative to the corresponding three-photon cross-section (as illustrated in Fig. 3) would allow efficient photo-activation of Indo-1 using two-photon excitation at 700 nm with an approximate 260-fold reduction in tissue absorbance relative to single-photon excitation at 350 nm (as illustrated in Fig. 8). As an alternate example, activation of Indo-1 in melanotic human tissue affords further illustration. In this example, the large absorbance of light at 700 nm by melanin indicates that use of longer wavelengths would be preferable to avoid matrix interferences and as such three-photon excitation of Indo-1

at 1000 nm would be optimal as a consequence of the approximate 15-fold reduction in tissue absorbance of light at 1000 nm relative to 700 nm and the approximate 3500-fold reduction in tissue absorbance of light at 1000 nm relative to 350 nm.

It should be noted that in general the most desirable wavelengths for multi-photon excitation will fall between 500 nm and 4000 nm as a consequence of the favorable tissue transmission properties in this band. Moreover, in addition to spectroscopic issues (such as tissue and agent absorbance properties), the order of a particular multi-photon process can be further chosen to match spatial localization performance to that which is optimal for the particular specimen or target to be examined. For example, in general, higher-order multi-photon processes (≥ 3 photons) will provide greater spatial localization than that observed with two-photon excitation. Thus for certain specimens, it may be desirable to use such higher order excitation processes for agent photo-activation, even if this may result in reduced detection sensitivity.

Excitation sources for multi-photon photo-activation:

The cross-section for a particular multi-photon excitation process is typically many-fold smaller than that for an equivalent single-photon excitation process yielding the same activated state as the multi-photon process. This is due to the relatively low probability that two or more photons will interact with an agent in a substantially simultaneous manner. However, it is preferable when using the present invention to employ the optical excitation sources capable of providing high instantaneous irradiance, such as for example mode-locked lasers (including titanium:sapphire lasers and Nd:YAG lasers) and amplified mode-locked lasers (including the regeneratively amplified titanium:sapphire laser and chirp-pulse amplified Nd:YAG lasers), can substantially ameliorate the impact of this low efficiency by increasing the incident instantaneous irradiance and thereby dramatically increase the effective efficiency of multi-photon excitation. Such lasers typically offer ultrashort pulsed output (having pulse widths ranging from 10 fs to 10 ps) at high pulse repetition rates (ranging from 1 kHz to 100 MHz) with modest average powers (ranging from 1 mW to 10 W). Such output properties facilitate selective, efficient multi-photon excitation, since the high instantaneous irradiance obtainable is capable of stimulating multi-photon processes while the short pulse widths and modest average powers minimize undesirable photo-activation of surrounding media. For example, when using continuous

5 wave excitation, the efficiency of three-photon excitation for a particular agent may be a factor of 10^7 or more smaller than that achievable with single-photon excitation. However, if the same average optical power is emitted in the form of a train of ultrashort pulses, the shift in production of the instantaneous and average irradiance can change this ratio such that it is close to unity. The special properties of ultrashort pulsed excitation allows this dramatic improvement without concomitant increases in collateral damage.

10 Note that sources capable of emitting relatively low energy pulses (such as for example the mode-locked titanium:sapphire laser, with typical pulse energies in the range of 1-10 nJ) are optimally suited to photo-activation of diagnostic or imaging agents using two- or more photons under focused illumination conditions, while sources capable of emitting relatively high energy pulses (such as for example the regeneratively amplified titanium:sapphire laser, with typical pulse energies in the range of 1-10 μ J) are optimally suited to photo-activation of such agents using two- or more photons under non-focused illumination conditions, for example to activate agents over a large area or within a large volume of tissue. Both are intended to be included within the scope of the present invention. This description describes possible optical excitation sources and is not intended to limit the present invention using only the optical excitation sources discussed above, as it is contemplated that other optical excitation sources could also be used within the scope of the present invention.

Detection of multi-photon excited emission from diagnostic and imaging agents:

20 Spatial information concerning the origin of the emitted light from a multi-photon excited diagnostic imaging agent is encoded by and may be correlated to the excitation focus. This is in stark contrast with single-photon excited imaging methods, including those based on photon migration, where the diagnostic imaging signal must be carefully deconvolved from emission light generated along the entire excitation path and from emission produced by scattered excitation light. Hence, it is not necessary for the light emitted from the multi-photon excited diagnostic agent to be detected or imaged directly without scatter. In fact, it is only necessary that a fraction of this emitted light be collected and detected in such a way that the collection and detection process does not distort the correlation between detected signal and emission point of origin.

More specifically, multi-photon imaging requires far less light for detection than is needed for excitation. Hence, a loss of light from scattering during emission is acceptable. Further, the emitted light only comes from a well defined location (the region of excitation), making it easier to selectively detect. Preferably, the present invention uses agents that emit at relatively long wavelengths. The present invention, however, is not limited to such agents.

To understand the significance of the relationship between signal detection and multi-photon excited emission point of origin, it is useful to consider what happens to the emitted light immediately following the instant of emission. When imaging in an optically dense specimen, such as biological tissue, light from the multi-photon excited diagnostic imaging agent will be emitted in an essentially isotropic manner. Some fraction of this emitted light will travel directly to a detector apparatus mounted remotely from the point of emission, while some other fraction will travel a circuitous route to the detector apparatus as a consequence of one or more scattering events occurring between emission and detection. If an attempt is made to image at a depth of 10 cm in a biological specimen, the transit time for an unscattered, or ballistic, emitted photon (that is, the total transit time from instant of emission to exit from a surface of the specimen) will be approximately 0.3 ns. For a highly scattered emitted photon, this transit time could be as high as 3-10 ns. Thus, for maximum efficiency in this example, it would be desirable to integrate all of the emitted light for a period of time sufficient to capture most or all of the ballistic and highly scattered photons. This implies that for imaging at depths of 10 cm or less, an integration period of approximately 10 ns would be appropriate.

If an image is to be generated by moving or scanning the location of the excitation focus relative to the specimen, the foregoing analysis implies that the excitation point should not be moved more frequently than once every 10 ns. In fact, practical limitations on scanning processes and mechanisms, combined with signal-to-noise arguments concerning minimum dwell times and the additional possible use of modulation methods, mandate that scanning be performed using dwell times typically in excess of 1 μ s. Thus, for example, for intensity based imaging with dwell times in excess of 1 μ s and possible modulation frequencies of 100 MHz or less, it makes little difference where the detector is located as long as it is situated such that it can collect a significant portion of the ballistic and scattered emitted light. The choice of location of the detector relative to the

emission point of origin, and hence the length of time introduced due to optical delay, has little or no effect on the ability to correlate the detected signal with its origin because of the short transit time relative to other measurement parameters. Accordingly, it will be clear that the detector may be located in such a way that it comprises an epi-illumination configuration with the excitation beam, or that it may be located externally to the excitation beam. It is notable that the epi-illumination configuration (or other possible co-linear excitation and detection configurations) minimizes potential parallax losses for detection of surface or near surface objects, but that such configurations are more susceptible to interference from elastically scattered or reflected excitation light. Parallax losses may be minimized for external detection configurations by actively orienting the detection system such that it maintains consistent registry with the point of excitation, by using multiple detection assemblies that are individually optimized for collection of emitted light from different zones within the specimen, or by locating the detection system sufficiently far from the specimen such that parallax losses are minimal. Hence, imaging depends on knowing where the illuminated region is, unless the fluorescent agent is controlled as to where it goes, as illustrated in Fig. 12.

The discussion on detection of emitted light from multi-photon excited diagnostic imaging agents has focused to this point on intensity based methods, wherein an image may be constructed by correlating detected intensity of emission with location of excitation for multiple excitation points throughout a specimen. However, intensity based methods are not always optimal, since they are susceptible to a number of complicating factors, including:

- Variations in scatter and absorption of excitation light due to heterogeneities in the specimen – heterogeneities, such as areas of abnormal optical density, that are located between the excitation source and the intended point of excitation can translate into unanticipated differences in effective excitation level at the intended point of excitation. Artifacts caused by this phenomenon can be ameliorated by acquiring data along several excitation paths that are affected to different extents by this heterogeneity, followed by subsequent deconvolution of the resultant multiple data sets, but this may be difficult or impossible for some specimens.
- Variations in scatter and absorption of emitted light due to heterogeneities in the specimen – heterogeneities, such as areas of abnormal optical density, that are located

between the point of emission and the detection system can translate into unanticipated differences in collection efficiency for light emitted from the point of excitation. Artifacts caused by this phenomenon can be ameliorated by acquiring data along several collection paths that are affected to different extents by this heterogeneity, followed by subsequent deconvolution of the resultant multiple data sets, but this may be difficult or impossible for some specimens.

- Variations in concentration or local environment of diagnostic imaging agents that are not directly correlated with form or function – it is assumed in intensity based imaging that changes in emission level throughout a specimen can be correlated with structural or physiological organization of the specimen. However, if the imaging agent is not appropriately distributed throughout the specimen, or if other factors, such as heterogeneity in the local environment within the specimen, affect the emission of the imaging agent in ways that cannot be correlated with form or function, then it becomes harder to obtain meaningful data from the specimen. Artifacts caused by this phenomenon can be ameliorated by using or by designing imaging agents that are not susceptible to such factors, but this may be difficult or impossible for some specimens.

A detection approach that is less susceptible to optical heterogeneity of the specimen could be based on measurement of change in excited state lifetime rather than on intensity of emission. Excited state lifetimes are an intrinsic property of the excited state of a molecular agent and its immediate environment (and, notably, are unrelated to the mechanism or method used for photo-activation). Fortuitously, the accurate measurement of lifetimes are immune to all but the grossest variations in excitation level and collection efficiency. A convenient way for measuring excited state lifetimes uses phase photometric methods to correlate phase shift between a modulated excitation source and the resultant emission signal to lifetime. Specifically, the preceding discussion on photon transit times implies that phase photometric methods are applicable for imaging in optically dense media, especially for agents with lifetimes in excess of 1-10 ns. Hence, if diagnostic imaging agents are used that have emission lifetimes that correlate with form or function within the specimen, such as quenching of fluorescence of an imaging agent in the presence of oxygen or concentration of an imaging agent within a structure, then imaging based on change in lifetime rather than on emission intensity becomes practical. Such

lifetime based methods would have equal applicability to laser scanning microscopy and to remote imaging of extended objects, such as a tumor in a human subject.

Appropriate collection devices for transduction of intensity or phase based emission data include, but are not limited to, photomultiplier tubes, microchannel plate devices, photodiodes, avalanche photodiodes, charge coupled devices and charge coupled device arrays, charge injection devices and charge injection device arrays, and photographic film.

Noise reduction methods for recovery of multi-photon excited emission from diagnostic imaging agents – modulation and harmonic detection:

The inherently low efficiency of the multi-photon excitation process can translate into a very high ratio of scattered, unabsorbed excitation light to multi-photon excited emission. Furthermore, the importance of other possible linear interferences attributable to the use of high excitation power levels, including single-photon excited fluorescence of the agent or other species present in the specimen under examination, Raman scatter, and other phenomena, along with the need to eliminate interferences from ambient light and other optical or electronic noise sources, all indicate that a modulated excitation method coupled with appropriate demodulation of the detector signal should provide optimal discrimination against interferences and enhanced recovery of the analytical signal. In fact, background interferences, reported to be disadvantageous in Denk et al. U.S. Patent No. 5,034,613 could be largely circumvented if suitable modulation and demodulation methods were used, including demodulation at the pulse repetition frequency of the laser. In fact, use of such methods would dramatically improve signal-to-noise (SNR) performance in a microscope, such as for example, the microscope disclosed in Denk. In general, modulation can improve detection performance for virtually any measurement in one or more of the following ways:

(1) Rejection of continuous background or noise sources. For example, when using a microscope, such as Denk's two-photon laser scanning microscope, modulation of the excitation source with subsequent demodulation of the detector signal, using a device such as a lock-in amplifier (LIA) or a heterodyne demodulator, would limit detection system response to a band of frequencies closely related to the modulation frequency. By controlling the phase sensitivity of this demodulation, additional discrimination would be achieved against signals that are not linked to or closely matched with the modulation

pattern. Hence, by suitable selection of modulation frequency and demodulation phase, interferences from noise sources such as room light or electronic noise at specific frequencies, for example from a nearby electric motor, can be strongly rejected. This approach is equally valid for remote imaging of extended objects, such as a tumor in a human subject.

(2) Rejection of broadband or “pink noise” sources. The measurement environment, along with the electronics and other devices used for any measurement, contribute broadband noise, sometimes called pink noise, into any measurement. The impact of this intrinsic noise can be greatly reduced through the use of bandwidth-limited detection methods. Specifically, for a given optical measurement, the observed signal voltage, V_{SIGNAL} , resulting from the detected optical emission of a photo-activated analyte, is related to a detector input current, i_{INPUT} , produced by photons interacting with a detector, multiplied by the input impedance, Z_{INPUT} , and the gain of the detection system, G , according to the following:

$$V_{\text{SIGNAL}} = i_{\text{INPUT}} \cdot Z_{\text{INPUT}} \cdot G, \quad (4)$$

while the observed noise voltage, V_{NOISE} , may be approximated by the product of the noise current, i_{NOISE} , the input impedance, the square root of the electronic or optical bandwidth, B , of the detection system, and the gain, according to following:

$$V_{\text{NOISE}} = i_{\text{NOISE}} \cdot Z_{\text{INPUT}} \cdot B^{1/2} \cdot G. \quad (5)$$

Hence, the signal-to-noise ration (SNR) may be estimated from the ratio of these two voltages, ($V_{\text{SIGNAL}} / V_{\text{NOISE}}$). When a typical optical detector, such as a photomultiplier tube (PMT), is used to detect an unmodulated fluorescence signal, this detector will produce a certain signal level along with a noise current. For example, a standard PMT, such as the Hamamatsu R928 (7.4×10^5 A/W radiant anode sensitivity), with an optical input at a level of 10 pW produces $7.4 \mu\text{A}$ i_{SIGNAL} . If this signal current is converted to voltage in a low noise amplifier having a gain of 100, an input impedance of 50Ω , an input noise level of $5 \text{ nV}/\sqrt{\text{Hz}}$, and a bandwidth of 1 MHz, the following signals are produced:

$$V_{\text{SIGNAL}} = 7.4 \mu\text{A} \cdot 50 \Omega \cdot 100 = 37 \text{ mV}; \quad (5a)$$

$$V_{\text{NOISE}} = 5 \text{ nV}/\sqrt{\text{Hz}} \cdot (10^6 \text{ Hz})^{1/2} \cdot 100 = 0.5 \text{ mV}. \quad (5b)$$

Note that Ohm's Law, or $V = i \cdot R$, has been substituted for noise current and impedance shown in Eq. 5. Thus, for this broadband example, $SNR = 74$. If this excitation energy is modulated, for example sinusoidally at 1 MHz with a 100% depth of modulation, the value of V_{SIGNAL} will decrease to approximately 18.5 mV (assuming that this modulation is introduced by cyclic attenuation or other loss-based modulation method that results in an overall loss of 50% of average power without changing peak excitation power). But if the detection system uses bandwidth limited demodulation at 1 MHz having a bandwidth of 1 kHz, the pink noise decreases far faster than the signal:

$$V_{\text{NOISE}} = 5 \text{ nV}/\sqrt{\text{Hz}} \cdot (10^3 \text{ Hz})^{1/2} \cdot 100 = 16 \mu\text{V}, \quad (5c)$$

and the overall SNR increases to approximately 1200. Thus, although some absolute signal level is lost when using many forms of modulation, the overall increase in SNR more than compensates for this loss. Further, if there is any linear interference in the detector response, for example from ambient light leakage into the detector, the broadband detection scheme will detect this as an additional noise source, while the modulated, bandwidth limited scheme will reject this interference. Assume that ambient leakage produces a background signal of 1 μA on the PMT, which translates to 5 mV of background signal. For the unmodulated case, optical shot noise from this background, B , is equal to the square root of the total photons detected, and $SNR \approx S/(S + B)^{1/2}$; this yields an estimated SNR of approximately 5.7. Notably, the SNR for the modulated case is essentially unchanged. This analysis is equally applicable to laser scanning microscopy and to remote imaging of extended objects, such as a tumor in a human subject.

(3) Rejection of linear interferences at the modulation frequency. As a consequence of the inherently low efficiency of multi-photon excitation, the ratio of scattered, unabsorbed excitation light to multi-photon excited emission is generally quite high. This includes linear interferences at the modulation frequency that arise from elastic and inelastic scatter as well as from single-photon excited emission. Optical filtering is

frequently used in an effort to spectrally distinguish multi-photon emission from these optical background phenomena. Unfortunately, these interferences can be exceedingly difficult or impossible to eliminate using spectral means alone. As an alternative to ignoring these residual interference sources, one approach for recovery of a pure multi-photon signal utilizes regression of the detected signal at several excitation power levels against the excitation power level, so that the non-linear multi-photon excited component can be extracted mathematically from linear interferences. This makes use of a model of total fluorescence response, I_f , given by:

$$I_f = \alpha I_L + \beta I_L^n \quad (6)$$

where I_L is the instantaneous excitation intensity, α is a proportionality constant for various linear effects, β is a proportionality constant for multi-photon excited emission, and n is the number of photons used for photo-activation.

While this regression-based method is appropriate for laboratory use where the necessary number of measurements per unit of time is small, it is too time consuming, complicated, and impractical whenever total data acquisition time must be minimized, such as in the case of multiple point scanned optical imaging which could be used for cancer or tumor detection. Far faster results can be obtained through the use of temporal rejection methods, such as second harmonic detection, which eliminates the need for performing multiple measurements at several power levels. Freeman et al. (R.G. Freeman, D.L. Gilliland and F.E. Lytle, "Second Harmonic Detection of Sinusoidally Modulated Two-Photon Excited Fluorescence," *Analytical Chemistry*, 62 (1990) 2216-2219) discuss second harmonic detection methods useful for the analysis and characterization of chemical samples in test tubes, wherein sinusoidal modulation of the excitation source is used to generate a signal at twice the modulation frequency that is related only to two-photon excited fluorescence. Freeman, however, does not appear to be used for imaging. A lock-in amplifier referenced to the modulation frequency is used to recover the pure two-photon signal at the second harmonic of the modulation frequency. While the second harmonic fluorescence signal is only approximately 12% of the total two-photon fluorescence produced, the improved rejection of linear interferences more than compensates for the loss in absolute signal level, resulting in an increase in the overall

SNR. Hence, the second harmonic detection method is ideally applicable to laser scanning microscopy and to remote imaging of extended objects, such as a tumor in a human subject, as a consequence of its intrinsic efficiency in rejection of scatter and its high data bandwidth potential. These advantages mean that an imaging system using second harmonic detection can reliably obtain pure two-photon excited emission signals with minimal dwell times at each point, and with use of maximum excitation power for each measurement at each point.

For the more general case of multi-photon excitation, similar harmonic analysis may be used at the n th harmonic, where n is as defined in Eq. 6. The signal that contains only the n -photon excited response will be at the n th harmonic. This approach for demodulation provides a general and powerful means for avoiding linear interferences for various non-linear excitation processes.

The preceding enumerated advantages for the use of modulation methods in multi-photon excited diagnostic imaging apply equally well whether data is acquired based on measurement of emission intensity or excited state lifetime. In fact, lifetime measurements are most readily and sensitively measured using phase photometric methods that are based on determination of phase shifts between a modulation waveform and the detected signal. Hence, it is clear that modulation methods have important utility in the efficient detection of multi-photon excited phenomena, where they serve to eliminate interferences from ambient and instrumental noise sources as well as from scattering and other phenomena occurring within the specimen undergoing examination. For optically dense media, such as human tissue, the extremely high ratio of scattered, unabsorbed excitation light to multi-photon excited emission makes use of such methods vital. Hence, for clinical imaging applications or for multi-photon laser scanning microscopy, employment of modulation methods as described here will always be advantageous.

Contrast agents in multi-photon excited imaging – endogenous and exogenous agents:

The foregoing discussion has shown that multi-photon excitation can be used to effect important improvements in the specificity and depth of penetration for optically excitable molecular agents present in optically dense media, and that detection performance can be improved by use of encoding and decoding methods on the respective excitation and detection processes. The exceptional spatial localization of excitation possible when using

multi-photon methods can be harnessed to significantly improve contrast in the point of excitation. Once this localized excitation is effected, the analytic light thereby emitted may be detected using a variety of detection means. If this excitation point is moved relative to the specimen under examination, for example by scanning the position of the focus relative to the specimen or by scanning the position of the specimen relative to the focus, then a two- or three-dimensional image of the specimen can be generated by making a correlation between the location of the excitation point and the emitted light thereby produced. Useful contrast in this image, however, also depends on the existence of differences in the concentration or local environment of the molecular agent or agents responsible for emission. These agents may be endogenous or exogenous to the specimen, and imaging is ultimately based on contrasts in their localized emission properties that can be correlated to heterogeneity in structure or function within the specimen. Hence, the role of these contrast agents in non-linear diagnostics or imaging is also important.

Various endogenous chromophoric agents may be useful for diagnostics or imaging, particularly of diseased tissue. Because of structural or physiological differences between diseased and non-diseased tissues, between various internal substructures and organs in higher animals, or between different ranges of healthy or sub-healthy tissues, the concentration or local environment of natural chromophoric agents, such as aromatic amino acids, proteins, nucleic acids, cellular energy exchange stores (such as adenosine triphosphate), enzymes, hormones, or other agents, can vary in ways that are useful for probing structural or functional heterogeneity. Thus, these endogenous indicators of heterogeneity can be probed non-invasively using multi-photon excitation.

Unfortunately, in many cases, the specificity possible with such agents is inadequate to achieve meaningful results, and so exogenous agents must be added to the specimen. Traditional exogenous agents semi-selectively partition into specific tissues, organs, or other structural units of a specimen following administration. The route for administration of these agents is typically topical application or via systemic administration. Under ideal conditions, these agents will partition into or otherwise become concentrated on or in the structures of interest, or may be excluded preferentially from these structures. This concentration is possibly a consequence of isolated topical application directly onto a superficial structure, or through intrinsic differences in the physical or chemical properties

of the structure which lead to partitioning of the agent into the structure. Contrasts between areas of high concentration and low concentration can thereby be used as a basis for probing structural or physiological heterogeneity. Alternatively, exogenous agents may permeate throughout a specimen; if their emission properties, such as chromatic shift, quenching, or lifetime, are sensitive to physiological heterogeneity, then these parameters of the contrast agent can be used as the basis for contrast in imaging.

Because the emission properties of a molecular agent are determined by the fundamental properties of the excited state and its environment, the mechanism responsible for promoting the agent to the excited state has no significant impact on the emission properties of the excited state. Hence, a molecular diagnostic or contrast agent that works well under single-photon excitation conditions may be expected to exhibit similar behavior under multi-photon excitation conditions. In general, any contrast agent that is useful for single-photon excitation can be used with multi-photon excitation, where the enhanced control over the site of excitation will serve to improve resolution of the image. Appropriate contrast agents include many molecular agents used as biological dyes or stains, as well as those used for photodynamic therapy (PDT). Standard PDT agents have tissue specificities that in general are based on the combined chemical and physical properties of the agent and the tissue, such as a cancerous lesion. These agents are efficient absorbers of optical energy, and in many cases are luminescent. Examples of these agents include, but are not limited to:

- various psoralen derivatives;
- various porphyrin and hematoporphyrin derivatives;
- various chlorin derivatives;
- various phthalocyanine derivatives;
- various rhodamine derivatives;
- various coumarin derivatives;
- various benzophenoxazine derivatives;
- chlorpromazine and its derivatives;
- various chlorophyll and bacteriochlorophyll derivatives;
- pheophorbide a [Pheo a]; merocyanine 540 [MC 540]; Vitamin D; 5-amino-laevulinic acid [ALA]; photosan; pheophorbide-a [Ph-a]; phenoxazine Nile blue derivatives (including various phenoxazine dyes);

- various charge transfer and radiative transfer agents; and
- numerous other photo-active or photosensitizing agents.

These agents will in general become accumulated either at or near a point of application or semi-selectively within a specific tissue due to differences in the physical or chemical properties of the tissue which lead to partitioning of the PDT agent into the tissue. Once accumulated, such agents will be susceptible to multi-photon excitation, and their luminescent or other emission properties can then be used for acquisition of diagnostic or imagery data. Other photo-active agents that absorb light and are capable of subsequent energy transfer to one or more other agents may also be used, either alone or in conjunction with one or more responsive agents that are capable of accepting this transferred energy and transforming it into a radiative emission.

Biogenic contrast agents in multi-photon excited imaging

Under ideal conditions, standard contrast agents derive target specificity based on chemical or physical affinity for specific tissues. In this way, contrast agents partition into or otherwise become concentrated on or in tissues of interest. Unfortunately, this target specificity is usually not perfect. As a result, an improved method for increasing specificity in the targeting of agent destination is desired. One embodiment of the present invention to achieve such improvement in specificity is based on utilization of specific biological signatures of structure, function, or disease. For example, by coupling anti-sense oligonucleotide agents to one or more photo-active moieties, such as FITC, new biogenic contrast agents are created that are capable of selectively tagging only specific cells, such as cancerous cells, that contain complementary genetic encoding. Moreover, the basic approach is easily extended to numerous genetic-based diseases or other disorders by changing the oligomeric code used for the biogenic probe. Employment of multi-photon excitation enables this powerful approach to be applied using the combined bio-specificity of the biogenic probe and the high spatial localization inherent to the multi-photon photo-activation process. Thus, very high contrast, very high resolution imaging becomes possible at the genetic level using agents that are specifically targeted for a particular organ, tissue, or lesion.

An optimal design for biogenic probes utilizes one or more photo-active moieties that have emission properties that change upon complexation between the biogenic agent and

the target site. Specifically, changes in emission wavelength or lifetime upon complexation can be used to increase sensitivity of the general method, since such changes will help to increase contrast between areas containing complexed agent and those containing uncomplexed agent. An example is a biogenic agent based on a photo-active moiety that is quenched until complexation occurs, upon which occurrence emission becomes unquenched. Another example is an agent based on an intercalating photo-active moiety, such as psoralen, that is tethered to an anti-sense genetic sequence; upon complexation between the anti-sense sequence and its target sequence, intercalation of the photo-active moiety is enabled that leads to a chromatic shift in emission properties of the photo-active moiety.

It will be clear from the foregoing discussion that targeting methods based on other bio-specific modes, such as for example immunological, rather than solely on genetic, are also covered within the scope of the present invention. More specifically, agent specificity based on antigen-antibody methods, where an antibody probe is coupled to a photo-active group, provides a powerful new way for diagnosis of disease and infection. Additional ways for achieving biospecificity in agent targeting include, but are not limited to, use of DNA, RNA, amino acids, proteins, antibodies, ligands, haptens, carbohydrate receptors or complexing agents, protein receptors or complexing agents, lipid receptors or complexing agents, chelators, encapsulating vehicles, nanoparticles, short-or long-chain aliphatic or aromatic hydrocarbons, including those containing aldehydes, ketones, alcohols, esters, amides, amines, nitriles, azides, or other hydrophilic or hydrophobic moieties.

PREFERRED EMBODIMENTS OF THE PRESENT INVENTION

One of the preferred embodiments of the present invention is to employ the output of a high instantaneous irradiance, ultrashort pulsed source, such as for example a mode-locked titanium-sapphire laser or a regeneratively amplified titanium:sapphire laser, to induce multi-photon photo-activation of one or more endogenous or exogenous photo-active agents. This and other preferred embodiments are shown in FIGURES 10-12.

First Exemplary Embodiment of the Invention:

One specific preferred embodiment of the subject invention is to employ the output of a NIR source to induce multi-photon photo-activation of endogenous or exogenous diagnostic or imaging agents present in a specimen using light at a wavelength approximately twice, or more, than necessary for conventional single-photon photo-activation. This preferred embodiment is shown in FIGURE 10. The NIR source 108 produces a beam of NIR radiation 110 consisting of a rapid series of high peak power pulses of NIR radiation, and may consist, for example, of a standard commercially available mode-locked titanium:sapphire laser capable of outputting mode-locked pulses with durations <200 fs and pulse energies of up to about 20 nJ at pulse repetition frequencies in excess of about 10 MHz. Such a source produces a quasi-continuous beam of light having a relatively low average power (up to several watts) but high peak power (on the order of 100 kW) that is continuously tunable over a NIR wavelength band from approximately 690-1080 nm. The pulse train emitted by the NIR source 108 constitutes a beam of NIR radiation 110 that is easily focused using standard optical means, such as reflective or refractive optics 112. The focused NIR beam 114 can then be directed onto a specimen 116 to be imaged. Multi-photon photo-activation (e.g. two photon, three photon) of the diagnostic or imaging agent will be substantially limited to the focal zone 118 of the focused beam 114 due to the high instantaneous level that is only present at the focus. Excitation light that is scattered 120 by the specimen 116 will not have a sufficient instantaneous irradiance level for significant photo-activation of any diagnostic or imaging agent that may be present in areas outside of the focal zone 118. Light 122 emitted by diagnostic or imaging agent molecules present in the focal zone 118 will exit (light is emitted isotropically during fluorescence or phosphorescence) the focal zone 118 in a substantially isotropic manner. A portion of the emitted light 124 is captured by a detection apparatus 126, such as a photomultiplier tube, that is mounted at a position inside or outside of the specimen 116. This detection apparatus 126 is fitted with a wavelength selection apparatus 128, such as an optical bandpass filter, that serves to pre-process the captured portion of the emitted light 124 in such a way that the selection apparatus 128 rejects a major fraction of the elastically scattered light while passing a major fraction of light at the wavelength or wavelengths corresponding to that which is principally characteristic of emission from the diagnostic agent. The signal thus issued 130 from the detection apparatus 126 is captured by a processor 132, the primary purpose

of which is to record emission response from diagnostic or imaging agent as a function of location of the focal zone 118. By causing the location of the focal zone 118 to be scanned throughout the volume of the specimen 116, a complete image of the specimen 116 may be obtained by examining the contents of the processor 132 as a function of location of the focal zone 118. This image may be used to identify zones of interest 134, such as subcutaneous tumors or other diseased area.

Second Exemplary Embodiment of the Invention:

As an alternate to the above preferred embodiment, modulation apparatus may be incorporated into the general embodiment shown in FIGURE 10. Such modulation apparatus may be used to improve overall performance of the imaging system, such as to improve rejection of environmental or instrumental noise sources, to enable recovery of pure multi-photon excited emission, or to facilitate detection of emitted light using phase photometric approaches. Specifically, FIGURE 11 shows a modulator 126, such as an electro-optic or acousto-optic modulator, a chopper, or other apparatus, located so as to interact with the beam of NIR radiation 110 emitted by the NIR source 108 that can be used to encode the beam of NIR radiation 110 with a modulation pattern that is registered to the output of a modulator driver 138 that provides a drive signal 140 to the modulator 136. The modulated beam of NIR radiation 142 thereby produced is then directed onto the specimen 116 as described previously for FIGURE 10. The multi-photon excited emitted light 144 thereby produced will exit the focal zone 118 in an essentially isotropic manner. However, in contrast to the similar emitted light 122 described previously for FIGURE 10, this emitted light 144 will exhibit a modulation that is essentially synchronous with the modulation of the modulated beam of NIR radiation 142, which in turn is synchronous with the drive signal 140 issued by the modulator driver 138. A portion of the modulated emitted light 146 is captured by a detection apparatus 126, such as a photomultiplier tube, that is mounted at a position inside or outside of the specimen 116. This detection apparatus 126 is fitted with a wavelength selection apparatus 128, such as an optical bandpass filter, that serves to process the captured portion of the modulated emitted light 146 in such a way that the selection apparatus 128 rejects a major fraction of the elastically scattered light while passing a major fraction of light at the wavelength or wavelengths corresponding to that which is principally characteristic of

emission from the diagnostic agent. The modulated signal thus issued 148 from the detection apparatus 126 is captured by a processor apparatus 150. The processor 150 serves two primary purposes, first to demodulate the modulated signal thus issued 148 from the detection apparatus 126 using a demodulation reference output 152 issued by the modulator driver 138, and second to record the demodulated emission response from the diagnostic or imaging agent as a function of location of the focal zone 118. Hence, by causing the location of the focal zone 118 to be scanned throughout the volume of the specimen 116, a complete image of the specimen 116 may be obtained by examining the contents of the processor 150 as a function of location of the focal zone 118. This image may be used to identify zones of interest 134, such as subcutaneous tumors or other diseased areas.

Alternatively, the pulse frequency of the NIR source 108 can be used as a modulation source itself, producing a modulated beam of NIR radiation 142 at such pulse frequency. In this alternate embodiment, the source 108 serves the role of modulator 136 and modulator driver 138, and provides a source of the demodulation reference output 152 for the processor 150. As a result, no separate modulator or driver is needed.

Third Exemplary Embodiment of the Invention:

As a second alternate to this preferred embodiment, an unfocused beam of NIR radiation may be used to illuminate superficial features of a specimen to provide a direct imaging mode of detection. This is shown in FIGURE 12. Specifically, the output of a NIR source, such as for example a mode-locked titanium:sapphire laser, can be used to induce multi-photon photo-activation of endogenous or exogenous diagnostic or imaging agents present on or near the surface of a specimen using light at a wavelength approximately twice, or more, that necessary for conventional single-photon photo-activation. The NIR Source 108 produces a beam of NIR radiation 110 consisting of a rapid series of high peak power pulses of NIR radiation. This beam is modulated using a modulator 136 located so as to interact with the beam of NIR radiation 110 emitted by the NIR source 108. This modulator 136 encodes the beam of NIR radiation 110 with a modulation pattern that is registered to the output of a modulator driver 138 that provides a drive signal 140 to the modulator 136. The modulated beam of NIR radiation 142 thereby produced is then defocused using standard optical apparatus, such as

reflective or refractive optics 154, to produce a divergent excitation beam 156 that is directed onto a specimen 116 to be imaged. Multi-photon photo-activation of diagnostic or imaging agent present on or near the surface of the specimen 116 produces modulated multi-photon excited emitted light 144 having a modulation that is essentially synchronous with the modulation of the modulated beam of NIR radiation 142, which in turn is synchronous with the drive signal 140 issued by the modulator driver 138. A portion of the modulated emitted light 146 is captured by an imaging detection apparatus 158, such as a charge coupled device array, that is mounted at a position outside of the specimen 116. This imaging detection apparatus 158 is fitted with a wavelength selection apparatus 128, such as an optical bandpass filter, that serves to process the captured portion of the modulated emitted light 146 in such a way that the selection apparatus 128 rejects a major fraction of the elastically scattered light while passing a major fraction of light at the wavelength or wavelengths corresponding to that which is principally characteristic of emission from the diagnostic agent. The modulated signal thus issued 160 from the imaging detection apparatus 158 is captured by a processor 162. The processor 162 serves two primary purposes, first, to demodulate the modulated signal thus issued 160 from the imaging detection apparatus 158 using a demodulation reference output 152 issued by the modulator driver 138, and second, to record the demodulated emission response from the diagnostic or imaging agent as a function of the location of emission. Hence, this alternate embodiment enables direct videographic imaging of surface features 164, such as skin cancer lesions, to be performed based on spatial differences in multi-photon excited emission across the illuminated surface of the specimen 116.

Alternatively, the pulse frequency of the NIR source 108 as a modulation source itself, producing a modulated beam of NIR radiation 142 at such pulse frequency. In this alternate embodiment, the source 108 serves the role of modulator 136 and modulator driver 138, and provides a source of the demodulation reference output 152 for the processor 150. As a result, no separate modulator or driver is needed.

It will be clear that while the foregoing disclosure has primarily focused on example applications using multi-photon excitation of agents with ultrashort pulsed NIR optical radiation produced by mode-locked titanium:sapphire lasers, the present invention is not limited to such excitation nor to such narrowly defined optical sources. In fact, aspects of the present invention are applicable when optical excitation is effected using linear or

other non-linear methods. For example, various other optical sources are applicable, alone or in combination, such as continuous wave and pulsed lamps, diode light sources, semiconductor lasers; other types of gas, dye, and solid-state continuous, pulsed, or mode-locked lasers, including: argon ion lasers; krypton ion lasers; helium-neon lasers; helium-cadmium lasers; ruby lasers; Nd:YAG, Nd:YLF, Nd:YAP, Nd:YV04, Nd:Glass, and Nd:CrGsGG lasers; Cr:LiSF lasers; Er:YAG lasers; F-center lasers; Ho:YAF and Ho:YLF lasers; copper vapor lasers; nitrogen lasers; optical parametric oscillators, amplifiers and generators; regeneratively amplified lasers; chirped-pulse amplified lasers; and sunlight.

Such sources are capable of producing continuous or pulsed beams of light, for example with pulse repetition frequencies ranging from less than about 1 kHz to more than about 10 GHz, and pulse energies ranging from below about 10 picojoules to above about 50 millijoules, and are therefore particularly suited for use in the present invention.

Further, while the foregoing disclosure has focused on diagnostic and imaging applications for *in vivo* detection or characterization of disease in plant and animal tissue, it will also be clear that the present invention has additional utility whenever selective activation of a responsive target agent is desirable. Specifically, applications of certain aspects of the present invention in other fields, such as in biological microscopy and in other analytic methods and apparatus, are covered within the scope of the present invention. As an example, the use of modulation and demodulation methods as described herein have direct applicability in various forms of microscopy, such as single-photon laser scanning microscopy, two-photon laser scanning microscopy, and multi-photon laser scanning microscopy.

It will be understood that each of the elements described above, or two or more together, may also find useful application in other types of constructions or applications differing from the types described above.

While the present invention has been illustrated and described as embodied in a general method for improved selectivity in photo-activation of molecular diagnostic or imaging agents, it is not intended to be limited to the details shown, since it will be understood that various omissions, modifications, substitutions and changes in the forms and details of the method illustrated and in its operation can be made by those skilled in the art without departing in any way from the spirit of the present invention. For example, in the third

exemplary embodiment, the modulation and demodulation details may be omitted to produce a more simple imaging apparatus, although this example modification would yield an overall reduction in imaging performance.

This description has been offered for illustrative purposes only and is not intended to limit the invention of this application, which is defined in the claims below.

Without further analysis, the foregoing will so fully reveal the gist of the present invention that others can, by applying current knowledge, readily adapt it for various applications without omitting features that, from the standpoint of prior art, fairly constitute essential characteristics of the generic or specific aspects of this invention.

What is claimed as new and desired to be protected by Letters Patent is set forth in the appended claims.

We claim:

Claim 1. A method for diagnosis or imaging a particular volume of material, wherein the material contains at least one photo-active molecular agent, the method comprising the steps of:

(a) treating the particular volume of the material with light sufficient to promote multi-photon excitation of the photo-active molecular agent contained in the particular volume of the material;

(b) photo-activating at least one of the at least one photo-active molecular agent in the particular volume of the material, thereby producing at least one photo-activated molecular agent, wherein the at least one photo-activated molecular agent emits energy;

(c) detecting the energy emitted by the at least one photo-activated molecular agent; and

(d) producing a detected energy signal which is characteristic of the particular volume of material.

Claim 2. The method of Claim 1 wherein the light sufficient to promote multi-photon excitation of the at least one photo-active molecular agent is laser light.

Claim 3. The method of Claim 2 wherein the laser light comprises a train of one or more ultrashort pulses.

Claim 4. The method of Claim 3 wherein each of said one or more pulses has a duration of at most approximately 10 ps.

Claim 5. The method of Claim 1 wherein the light sufficient to promote multi-photon excitation of the photo-active molecular agent is a focused beam of light.

Claim 6. The method of Claim 5 wherein the focused beam of light is focused laser light.

Claim 7. The method of Claim 1 wherein the at least one photo-active molecular agent is an endogeneous agent.

Claim 8. The method of Claim 1 further including a first step of treating the material with at least one photo-active molecular agent, wherein the particular volume of the material retains at least a portion of the at least one photo-active molecular agent.

Claim 9. The method of Claim 8 wherein the at least one photo-active molecular agent is selected from the group consisting of psoralen, 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), 4,5',8-trimethylpsoralen (TMP), 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT), 5-chloromethyl-8-methoxypsoralen (HMT), angelicin (isopsoralen), 5-methylangelicin (5-MIP), 3-carboxypsoralen, porphyrin, haematoporphyrin derivative (HPD), photofrin II, benzoporphyrin derivative (BPD), protoporphyrin IX (PpIX), dye haematoporphyrin ether (DHE), polyhaematoporphyrin esters (PHE), 13,17-N,N,N-dimethylethylethanolamine ester of protoporphyrin (PH1008), tetra(3-hydroxyphenyl)-porphyrin (3-THPP), tetraphenylporphyrin monosulfonate (TPPS1), tetraphenylporphyrin disulfonate (TPPS2a), dihaematoporphyrin ether, mesotetraphenylporphyrin, mesotetra(4N-methylpyridyl)porphyrin (T4MpyP), octa-(4-*tert*-butylphenyl)tetrapyrazinoporphyrazine (OPTP), phthalocyanine, tetra-(4-*tert*-butyl)phthalocyanine (t_4 -PcH₂), tetra-(4-*tert*-butyl)phthalocyanatomagnesium (t_4 -PcMg), chloroaluminum sulfonated phthalocyanine (CASPc), chloroaluminum phthalocyanine tetrasulfate (AlPcTS), mono-sulfonated aluminum phthalocyanine (AlSPc), di-sulfonated aluminum phthalocyanine (AlS2Pc), tri-sulfonated aluminum phthalocyanine (AlS3Pc), tetra-sulfonated aluminum phthalocyanine (AlS4Pc), silicon phthalocyanine (SiPc IV), zinc II phthalocyanine (ZnPc), bis(di-isobutyl octadecylsiloxy)silicon 2,3-naphthalocyanine (isoBOSINC), germanium IV octabutoxyphthalocyanine (GePc), rhodamine 101 (Rh-101), rhodamine 110 (Rh-110), rhodamine 123 (Rh-123), rhodamine 19 (Rh-19), rhodamine 560 (Rh-560), rhodamine 575 (Rh-575), rhodamine 590 (Rh-590), rhodamine 610 (Rh-610), rhodamine 640 (Rh-640), rhodamine 6G (Rh-6G), rhodamine 700 (Rh-700), rhodamine 800 (Rh-800), rhodamine B (Rh-B), sulforhodamine 101, sulforhodamine 640, sulforhodamine B, coumarin 1, coumarin 2, coumarin 4, coumarin 6, coumarin 6H, coumarin 7, coumarin 30, coumarin 47, coumarin 102, coumarin 106,

coumarin 120, coumarin 151, coumarin 152, coumarin 152A, coumarin 153, coumarin 311, coumarin 307, coumarin 314, coumarin 334, coumarin 337, coumarin 343, coumarin 440, coumarin 450, coumarin 456, coumarin 460, coumarin 461, coumarin 466, coumarin 478, coumarin 480, coumarin 481, coumarin 485, coumarin 490, coumarin 500, coumarin 503, coumarin 504, coumarin 510, coumarin 515, coumarin 519, coumarin 521, coumarin 522, coumarin 523, coumarin 535, coumarin 540, coumarin 540A, coumarin 548, 5-ethylamino-9-diethylaminobenzo[a]phenoxazinium (EtNBA), 5-ethyl-amino-9-diethylaminobenzo[a]phenothiazinium (EtNBS), 5-ethylamino-9-diethylaminobenzo[a]phenoselenazinium (EtNBSe), chlorpromazine, chlorpromazine derivatives, chlorophyll derivatives, bacteriochlorophyll derivatives, metal-ligand complexes, tris(2,2'-bipyridine)ruthenium (II) dichloride (RuBPY), tris(2,2'-bipyridine)rhodium (II) dichloride (RhBPY), tris(2,2'-bipyridine)platinum (II) dichloride (PtBPY), pheophorbide a, merocyanine 540, vitamin D, 5-amino-laevulinic acid, photosan, chlorin e6, chlorin e6 ethylenediamide, mono-L-aspartyl chlorin e6, phenoxazine Nile blue derivatives, stilbene, stilbene derivatives, 4-(N-(2-hydroxyethyl)-N-methyl)-aminophenyl)-4'-(6-hydroxyhexylsulfonyl)stilbene (APSS), and standard biological dyes and stains.

Claim 10. The method of Claim 1 wherein said multi-photon photoactivation involves n photons, and wherein n equals 2 or more photons and can be varied so as to optimize the volume of tissue in which said agent is photoactivated.

Claim 11. The method of Claim 1 wherein the step of treating the particular volume of the material with light sufficient to promote multi-photon excitation of the at least one photo-active molecular agent contained in the particular volume of the material includes the steps of:

- (a1) modulating light from a light source, thereby producing a modulated light; and
- (a2) treating the particular volume of the material with the modulated light sufficient to promote multi-photon excitation of the at least one photo-active molecular agent contained in the particular volume of the material;

and further including the steps of:

- (e) demodulating the detected energy signal with the particular type of modulation;
and
(f) producing a demodulated energy signal which is characteristic of the particular volume of the material.

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Claim 12. The method of Claim 11 wherein said modulated light is modulated with a particular type of modulation and said detected energy signal includes said particular type of modulation.

Claim 13. A method according to Claim 11, wherein a microscope is used in the step of treating the particular volume of tissue with modulated light.

Claim 14. The method of Claim 12 wherein the step of demodulating the detected energy signal with the particular type of modulation includes demodulating the detected energy signal at a frequency twice or more that of the particular type of modulation, thereby detecting a harmonic of the particular type of modulation.

Claim 15. The method of Claim 11 wherein the demodulated energy signal which is characteristic of the particular volume of the material represents a change in lifetime of at least one photo-activated molecular agent present in the particular volume of the material.

Claim 16. The method of Claim 11 wherein said modulated light is produced by operating a laser to produce a pulsed output having a pulse repetition frequency above 10 megahertz and a sub-nanosecond pulse duration.

Claim 17. The method of Claim 1 wherein the treating step includes:
focusing a beam of light over a range of focal lengths so that a focal plane of the light beam extends to a location between a surface of the material and a point substantially beyond the material surface, whereby the treating step may extend to penetrate deep within the material.

Claim 18. The method of Claim 1, wherein said detected energy signal is used in forming an image of said particular volume of material.

Claim 19. The imaging method of Claim 18 wherein the treating with light step includes:

5 focusing a beam of light over a range of focal lengths so that a focal plane of the light beam extends to a location between a surface of the material and a point substantially beyond the material surface, whereby the treating step may extend to penetrate deep within the material,

10 further including varying, the focal length position of the light beam within the material, so that said steps of photo-activating, detecting, and producing a detected energy signal occur along varying positions between the material surface and a position located substantially beyond the material surface, whereby said image is three dimensional.

Claim 20. The method of Claim 2, wherein said laser light is produced by operating a laser to produce a pulsed output having a pulse repetition frequency above about 10 megahertz and a sub-nanosecond pulse duration.

Claim 21. The method of Claim 20 including operating the laser to produce near-infrared light.

Claim 22. The method of Claim 21 wherein the laser produces pulse energies of up to about 20 nanojoules.

Claim 23. The method of Claim 1 wherein said detecting step comprises detecting emitted light that does not retrace an optical path of the incident light from the laser.

Claim 24. The method the Claim 1 wherein said treating step further includes using a modulated laser light;

and wherein in the detecting step, a wavelength selection apparatus is used to filter energy emitted by said photo-activated agent.

Claim 25. The method of Claim 20 wherein said treating and photo-activating steps produce emitted light which is from the molecular agent in the material and said production of emitted light is substantially synchronous with a modulation of the laser light.

Claim 26. The method of Claim 1 wherein said material is plant or animal tissue.

Claim 27. The method of Claim 1 wherein said at least one photo-active molecular agent includes endogenous agents selected from the group comprising aromatic amino acids, nucleic acids, proteins, cellular energy exchange stores such as adenosine triphosphate, enzymes, and hormones.

Claim 28. The method of Claim 8 wherein said at least one photo-active molecular agent is coupled to a targeting agent before said step of treating the material with said at least one photo-active molecular agent, and wherein said targeting material is selected from the group comprising nucleic acids, amino acids, proteins, protein receptors, protein complexing agents, carbohydrates, carbohydrate receptors, carbohydrate complexing agents, lipids, lipid receptors, lipid complexing agents antibodies, ligands, haptens, complexing agents, chelators, encapsulating vehicles, liposomes, fullerenes, crown ethers, and cyclodextrins.

Claim 29. Apparatus for diagnosis or imaging a particular volume of material containing at least one photo-active molecular agent, the apparatus comprising:

a source of light, said light having a frequency effective to penetrate substantially into the materials, said light being adapted to promote multi-photon excitation of the molecular agent contained within the material;

focusing apparatus for focusing the light throughout a range of focal lengths extending from a surface of said material to a depth substantially beyond said surface, said light

source and focusing apparatus cooperating to promote multi-photon excitation of the molecular agent,

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wherein a focal point or focal plane is adjustable with respect to said material; and a detector positioned to detect said light emitted by the molecular agent and said detector configured to produce a detected signal characteristic of the particular volume at which the light source has been focused.

Claim 30. The apparatus of Claim 29 wherein said light source produces a pulsed output having a pulse repetition frequency in the range from about 1 kilohertz to about 10 gigahertz.

Claim 31. The apparatus of Claim 30 wherein said the pulse repetition frequency is above about 10 megahertz.

Claim 32. The apparatus of Claim 30 wherein said light source has a sub-nanosecond pulse duration.

Claim 33. The apparatus of Claim 29 wherein said light source produces near-infrared light.

Claim 34. The apparatus of Claim 30 wherein said light source operates within a range of pulse energies from about 10 picojoules to about 50 millijoules.

Claim 35. The apparatus of Claim 34 wherein said light source produces pulse energies of less than about 20 nanojoules.

Claim 36. The apparatus of Claim 33 wherein said light source comprises a laser.

Claim 37. The apparatus of Claim 36 wherein the light from the laser comprises a train of one or more ultrashort pulses.

Claim 38. The method of Claim 37 wherein each of said one or more pulses has a duration of at most approximately 10 ps.

Claim 39. The apparatus of Claim 29 further comprising a processor coupled to said detector.

Claim 40. The apparatus of Claim 39 further comprising a modulation system associated with said light source, said processor being coupled to said modulation system and configured to demodulate said detected signal.

Claim 41. The apparatus of Claim 29 wherein said multi-photon photoactivation involves n photons, and wherein n equals 2 or more photons and can be varied so as to optimize the volume of material in which said agent is photoactivated.

Claim 42. The apparatus of Claim 29 wherein the at least one photo-active molecular agent is an endogeneous agent.

Claim 43. The apparatus of Claim 29 wherein the at least one photo-active molecular agent is an exogeneous agent.

Claim 44. The apparatus of Claim 40 wherein said focusing apparatus is a microscope.

Claim 45. The apparatus of Claim 29 wherein said material is plant or animal tissue.

Claim 46. A method for medical diagnostic imaging comprising the steps of:
introducing a photo-active molecular agent into a tissue, said agent being selected for specificity of the tissue of interest, said agent being susceptible to multi-photon excitation;
allowing said agent to accumulate in specific tissue of interest;
directing light to specific regions of interest within the tissue, including regions substantially below a tissue surface, said light being selected in frequency and energy to

penetrate the tissue and to promote multi-photon excitation substantially only at a focal zone;

controlling the location of the focal zone over a range of depths within said tissue;

using multi-photon excitation, photoactivating said agent over said range of depths within the tissue, thereby producing photo-activated agents at the focal zone, wherein the photo-activated molecular agent emits energy;

detecting the emitted energy; and

producing a detected energy signal that is characteristic of tissue at the focal zone.

Claim 47. The method of Claim 46 wherein said step of directing light includes generating near infrared light using a pulsed laser operating at short pulse widths and a high pulse repetition rate, and focusing said light into said tissue.

Claim 48. The method light of Claim 46 wherein said step of controlling the location comprises varying the position of the focal zone relative to the specific tissue under examination or varying the position of the tissue under examination relative to a fixed focal zone.

Claim 49. The method of Claim 46 wherein said method further includes modulating the light before it is incident on the tissue and demodulating the detected energy signal.

Claim 50. The method of Claim 46 wherein said detected energy signal is used in forming an image of said focal zone.

Claim 51. Apparatus for medical diagnostic imaging comprising:

a light source, said light source producing light directed to or into tissue to be imaged, said light being selected in frequency and energy to penetrate into or below a surface of the tissue and to promote multi-photon excitation substantially only in a region to be imaged;

a focusing apparatus, said focusing apparatus being able to vary the position of the light within a range of depths in the region of tissue to be imaged;

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a detector positioned to receive and detect radiation emitted by a photo-activated molecular agent within the material after said agent has been excited using multi-photon excitation.

Claim 52. An apparatus according to Claim 51 wherein said light source produces near-infrared light.

Claim 53. An apparatus according to Claim 52 wherein said light source is a laser.

Claim 54. An apparatus according to Claim 51 further comprising a modulator, said modulator cooperating with said light source to modulate said light wherein said light is modulated with a type of modulation to produce a modulated light.

Claim 55. An apparatus according to Claim 54 further comprising a demodulator, said demodulator coupled to said detector and producing a demodulated energy signal which is characteristic of the particular photo-activated molecular agent.

Claim 56. An apparatus according to Claim 51 wherein said light source produces a pulsed output having a pulse repetition frequency above about 10 megahertz and a subnanosecond pulse duration.

Claim 57. An apparatus according to Claim 51 wherein said light source produces pulse energies of less than about 20 nanojoules.

Claim 58. An apparatus according to Claim 51 wherein said multi-photon photoactivation involves n photons, and wherein n equals 2 or more photons and can be varied so as to optimize the volume of tissue in which said agent is photoactivated.

Claim 59. A method for characterizing a material, the material including at least one photo-active molecular agent, the method comprising:

encoding light from a light source with a modulation pattern to produce a modulated light;

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treating the material with said modulated light to promote multi-photon excitation of said at least one photo-active molecular agent so that said at least one excited molecular agent becomes photo-activated in said material and emits a modulated energy;

detecting a portion of the modulated emitted energy; and

producing a detected modulated energy signal which is characteristic of the material.

Claim 60. The method of Claim 59 further comprising the steps of demodulating the detected modulated energy signal.

Claim 61. The method of Claim 59 wherein said method of characterizing is for use in imaging said material.

Claim 62. The method of Claim 59 further comprising the steps of:
demodulating the detected modulated energy signal; and
recording said demodulated energy signal as a function of the location of said at least one photo-activated molecular agent.

Claim 63. The method of Claim 59 wherein said material is selected from the group consisting of plant tissue and animal tissue.

Claim 64. The method of Claim 63 wherein said animal tissue is located in the body of the animal.

Claim 65. The method of Claim 59 wherein said light source is a laser.

Claim 66. The method of Claim 59 further including a step of treating the material with at least one photo-active molecular agent before treating said material with light.

Claim 67. The method of Claim 59 wherein a microscope is used in the step of treating the material with modulated light.

Claim 68. The method of Claim 59 wherein said multi-photon photoactivation involves n photons, and wherein n equal two or more photons and can be varied so as to optimize the volume of material in which said agent is photactivated.

Claim 69. A method for characterizing a particular volume of tissue, wherein the tissue includes at least one photo-active molecular agent, the method comprising:

encoding light from a light source with a modulation pattern to produce a modulated light;

5 directing light to specific regions of interest within the tissue, including regions substantially below a tissue surface, said light being selected to penetrate the tissue and to promote multi-photon excitation substantially only at locations within a focal zone;

controlling the locations of said focal zone over a range of depths within said tissue;

10 using multi-photon excitation, photo-activating said agent over said range of depths within said tissue, so that said at least one excited molecular agent becomes photo-activated substantially only at the focal zone, wherein said photo-activated agent emits a modulated energy;

detecting a portion of the emitted modulated energy; and

producing a detected modulated energy signal which is characteristic of the tissue.

Claim 70. The method of Claim 69 further comprising the step of demodulating the detected modulated energy signal.

Claim 71. The method of Claim 69 wherein said method of characterizing is for use in imaging said material.

Claim 72. The method of Claim 70 further comprising the step of recording said demodulated energy signal as a function of the location of said focal zone.

Claim 73. The method of Claim 69 wherein said tissue is located in the body of an animal.

Claim 74. The method of Claim 69 wherein said light source is a laser.

Claim 75. The method of Claim 69 further including a step of treating the tissue with at least one photo-active molecular agent before treating said tissue with light.

Claim 76. A method for the diagnostic characterization of tissue, the tissue having a surface, the tissue being relatively transparent to light having preselected characteristics, the method comprising the steps of:

introducing a selected photo-active agent into a tissue, said agent being susceptible of multi-photon excitation,

allowing said agent to accumulate at features of interest, if any, within said tissue;

operating a laser to obtain therefrom a beam of light having said preselected characteristics;

directing said laser beam to specific regions of interest within the tissue, including regions substantially below the tissue surface, including penetrating the tissue with said beam and promoting multi-photon excitation of said agent substantially only at locations within a focal zone;

moving the locations of said focal zone over a cross sectional area located at a range of depths within said tissue thereby to define an examined volume;

using multi-photon excitation, photo-activating any of said agent which has accumulated at any said feature of interest within said examined volume through which said focal zone passes, thereby producing a photo-activated agent at each said feature of interest when said focal zone intersects said feature of interest, wherein the photo-activated agent emits energy;

detecting the emitted energy; and

producing a detected energy signal that is characteristic of tissue at the focal zone.

Claim 77. The method of Claim 76 wherein said method for diagnostic characterization is for use in imaging said tissue.

Claim 78. The method of Claim 76 further including modulating said laser beam; wherein said photo-activated agent is caused to emit modulated energy, wherein said detecting step includes detecting modulated energy, wherein said producing step produces

5

a detected modulated energy signal; and using said detected modulated energy signal to form an image of any said features of interest in said tissue.

Fig. 1(c).

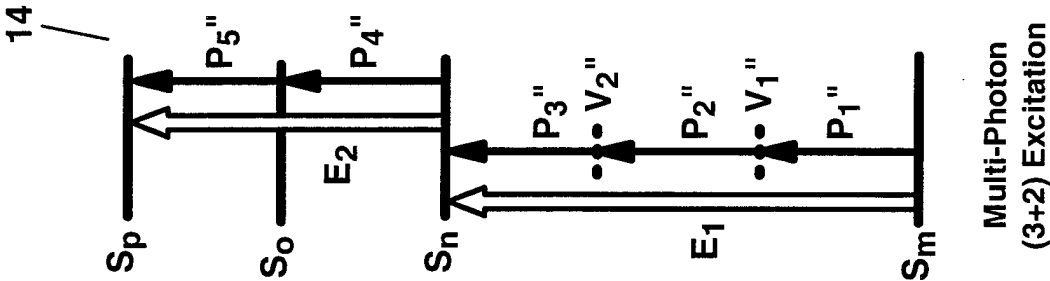


Fig. 1(b).

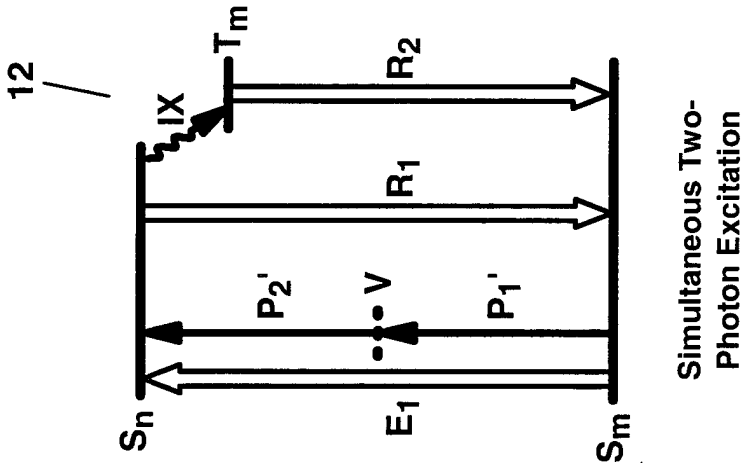


Fig. 1(a).

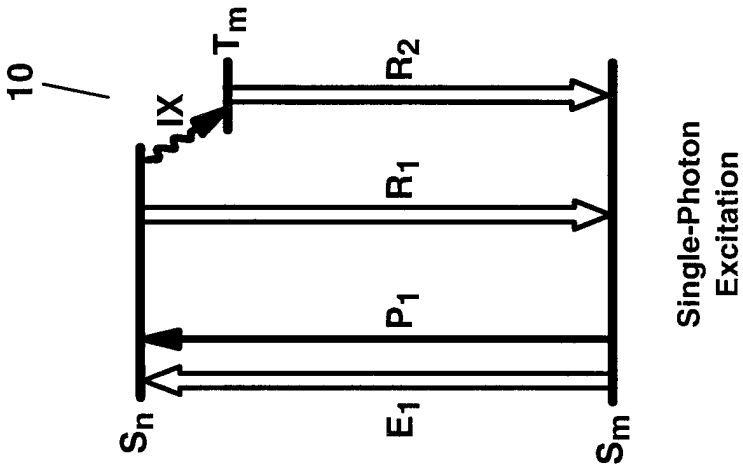


Fig. 2(a). **Fig. 2(b).** **Fig. 2(c).** **Fig. 2(d).**

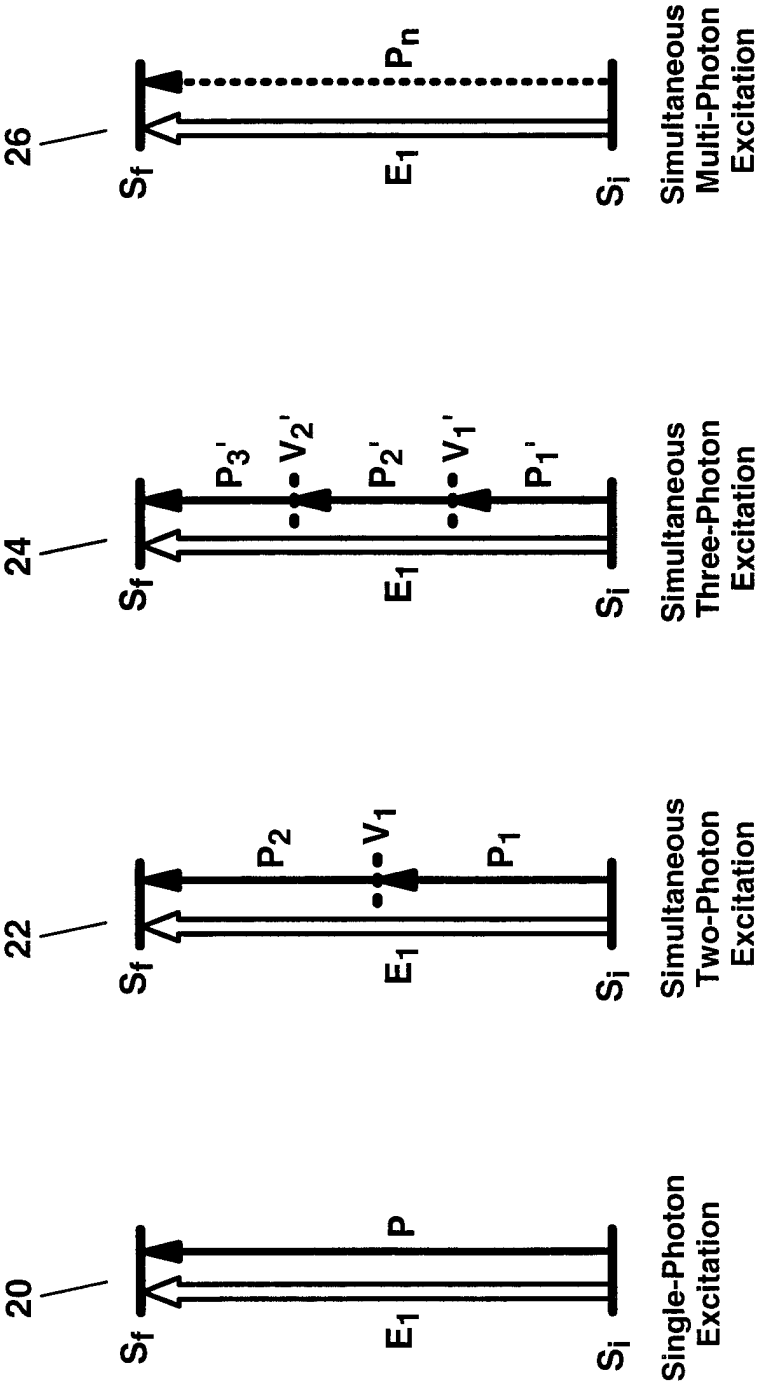


Fig. 3.

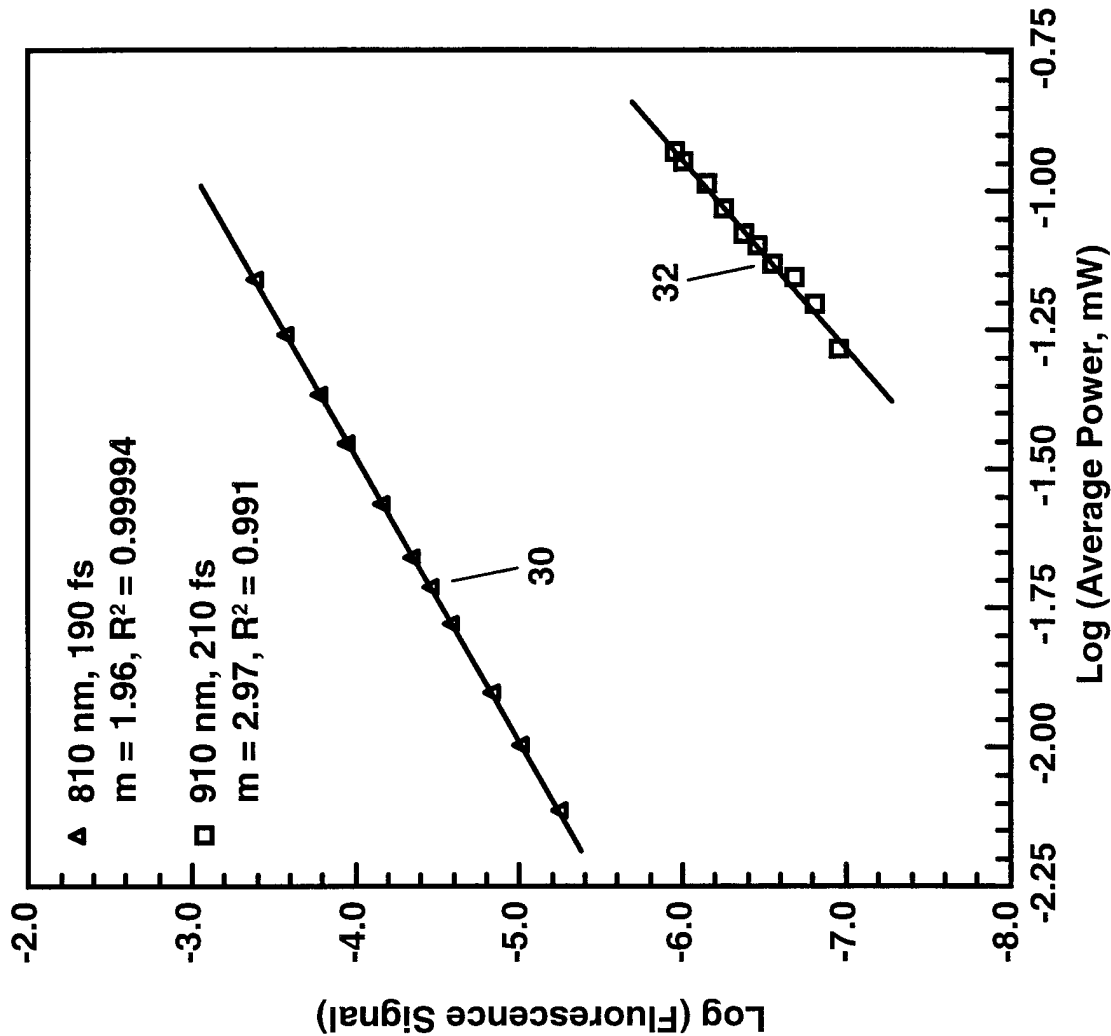


Fig. 4.

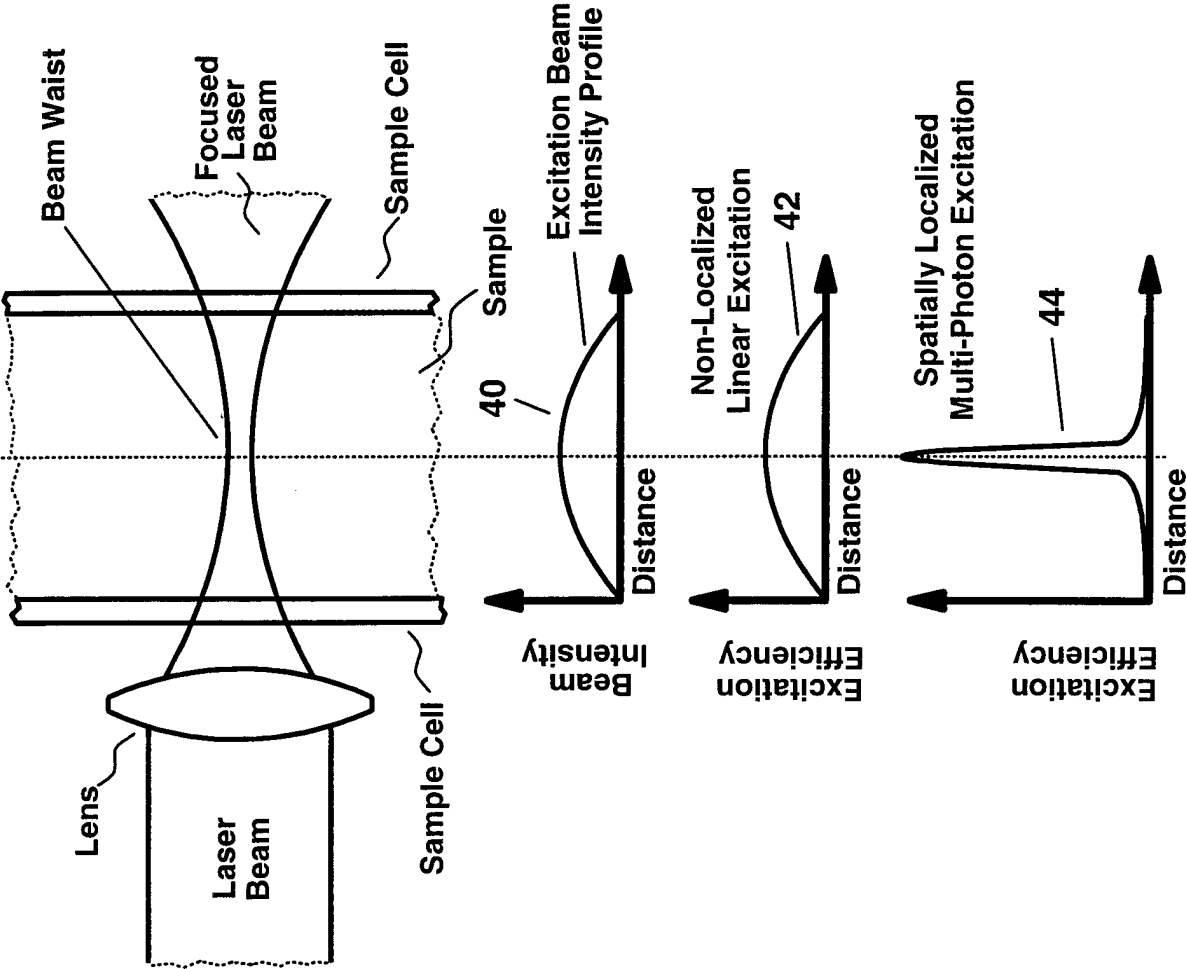


Fig. 5(a).

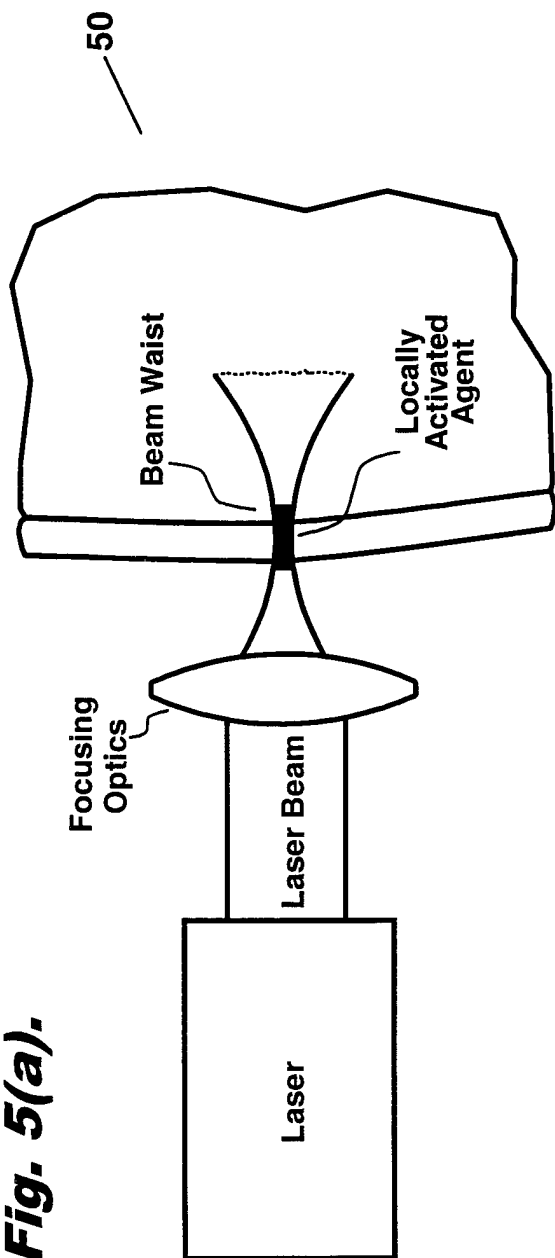


Fig. 5(b).

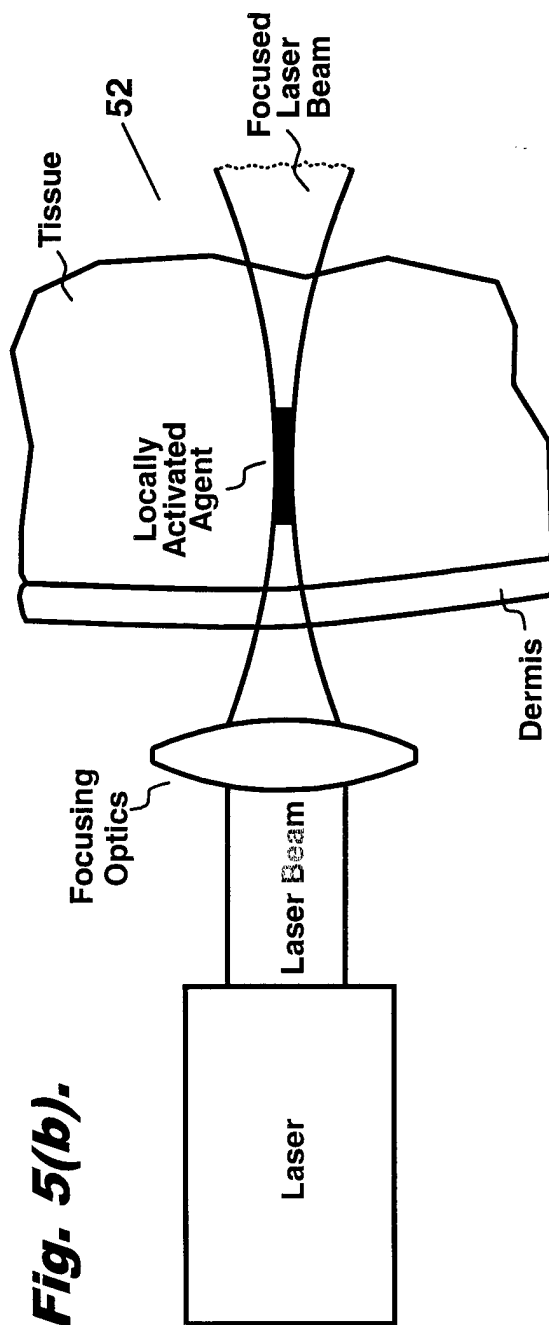


Fig. 6(a).

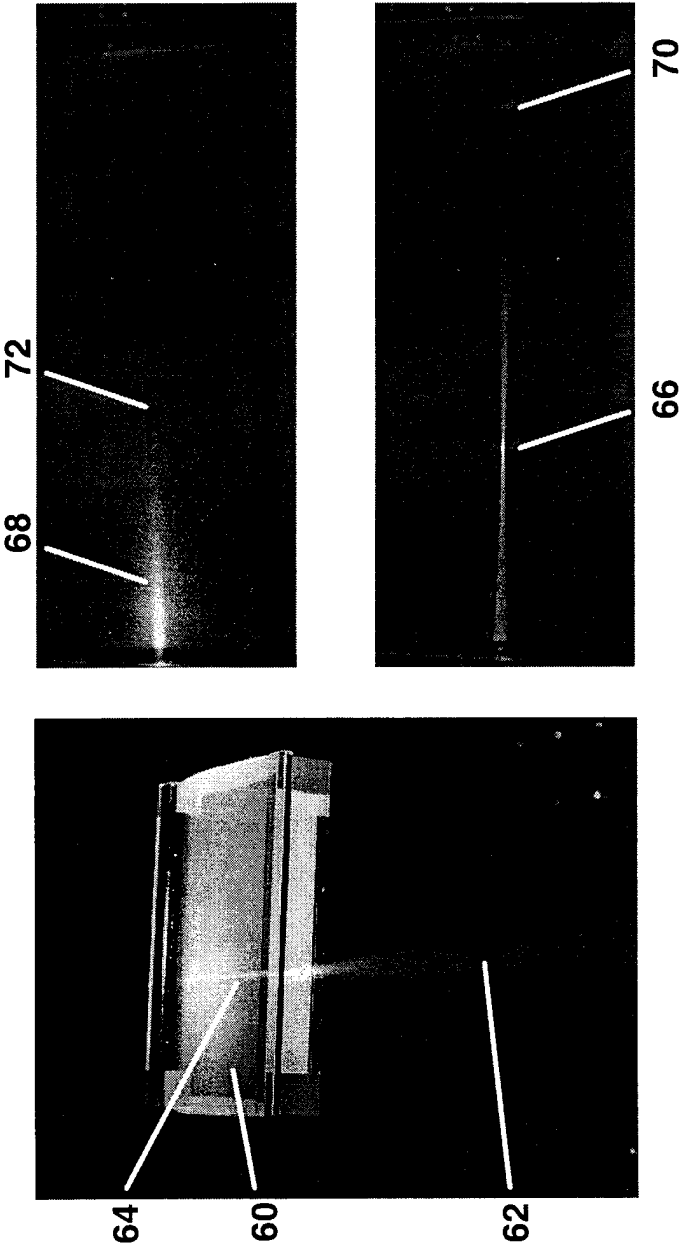


Fig. 6(b).

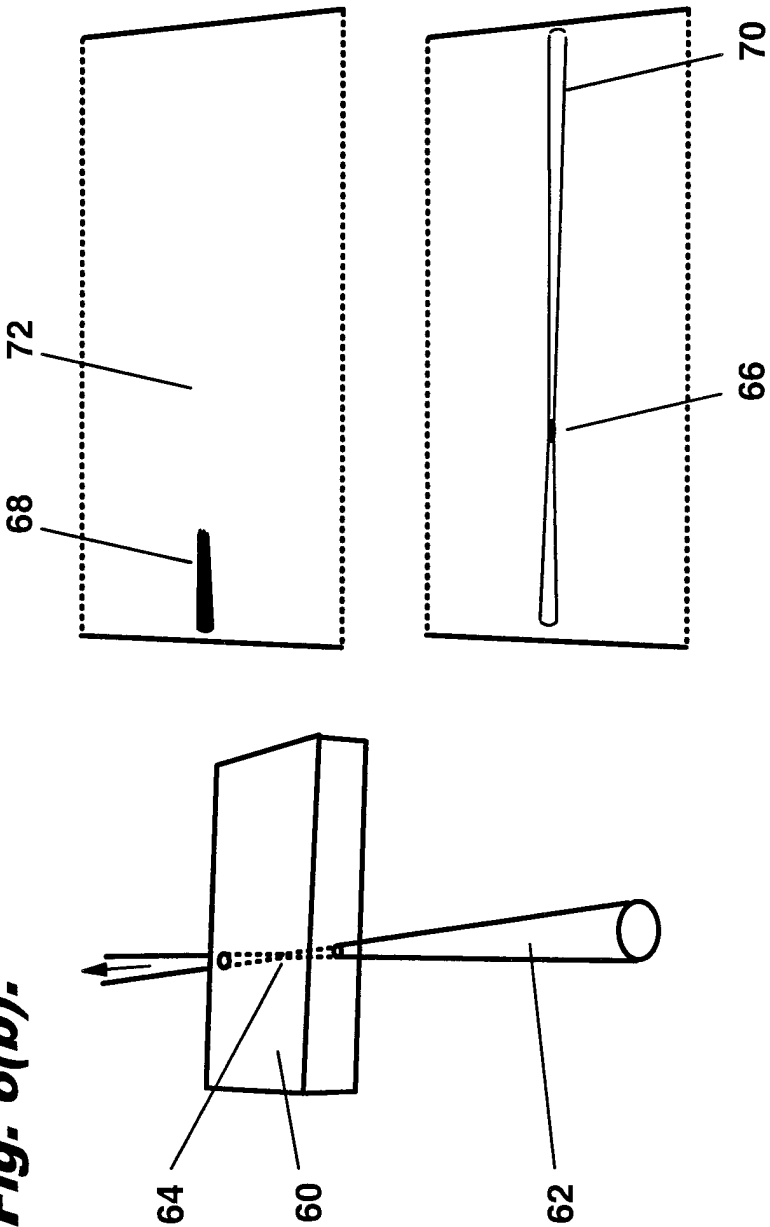


Fig. 7.

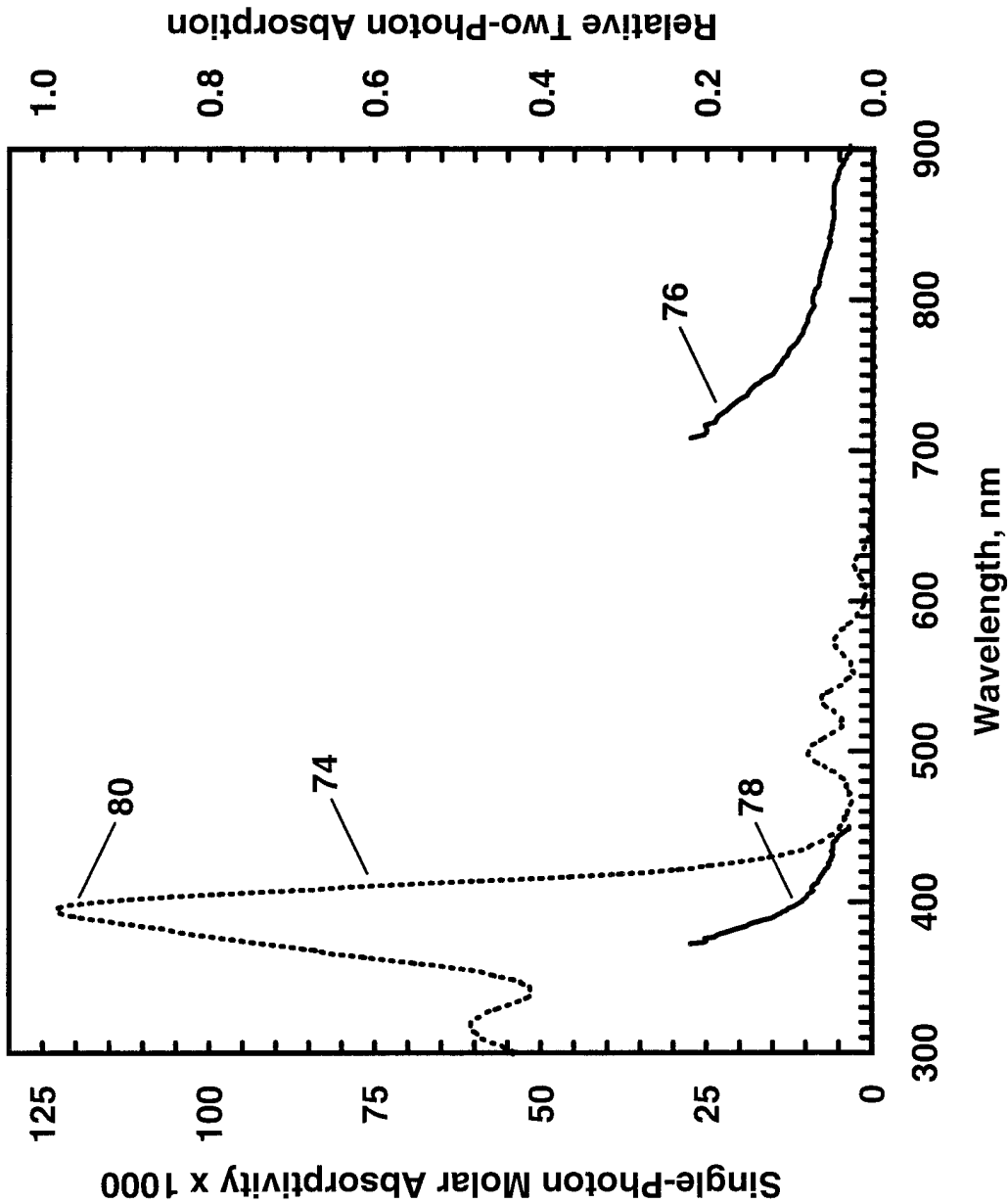


Fig. 8.

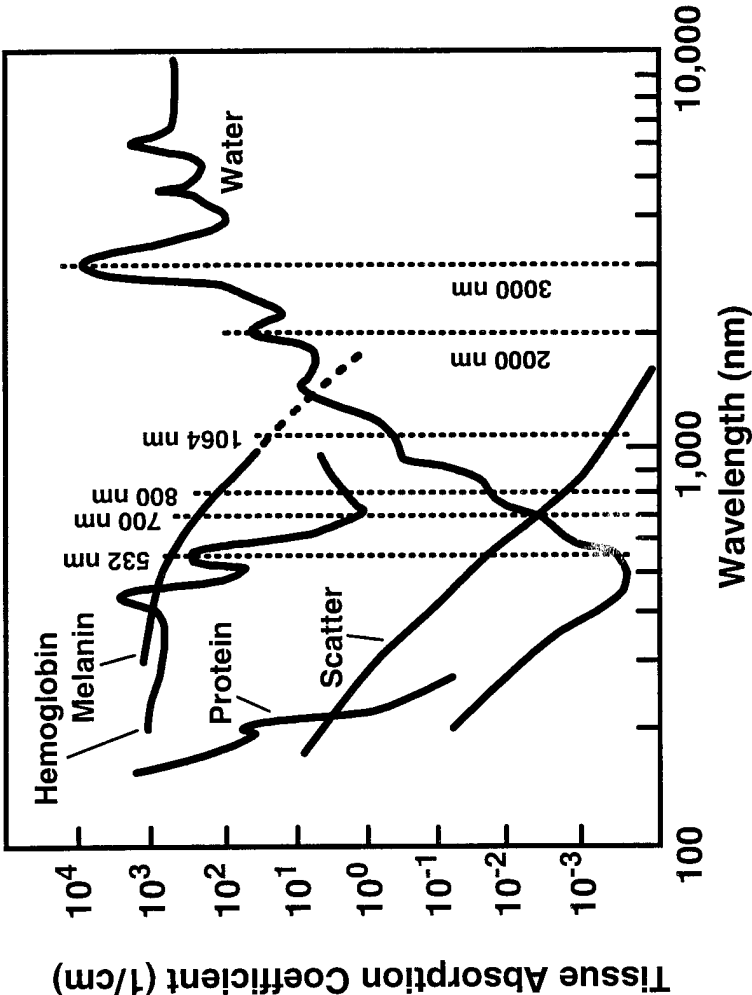
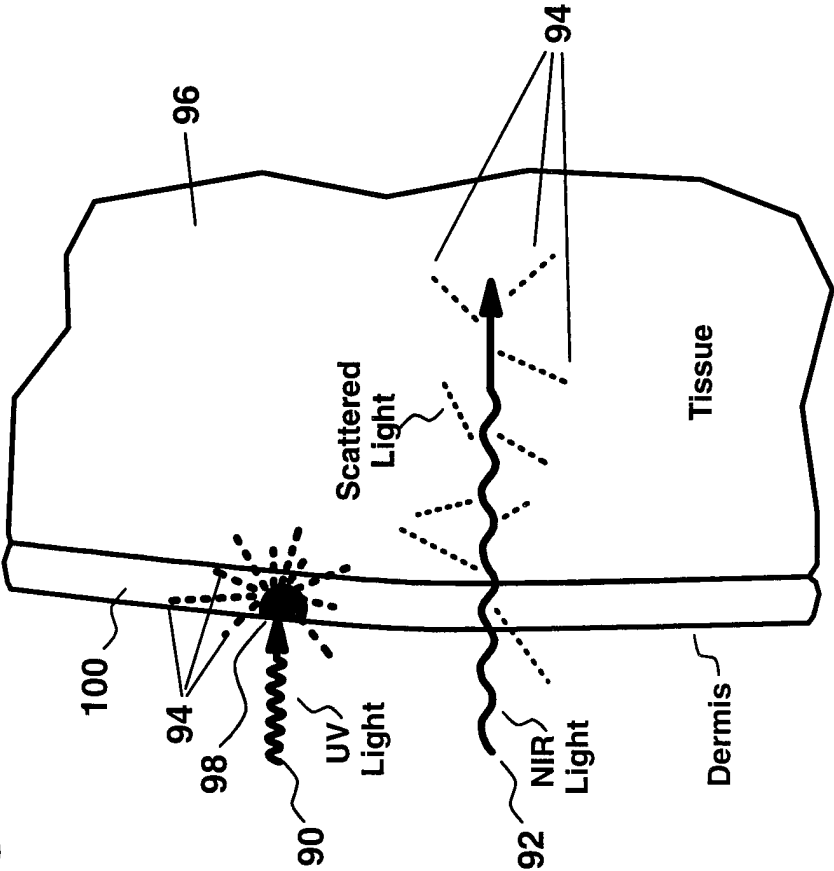


Fig. 9.



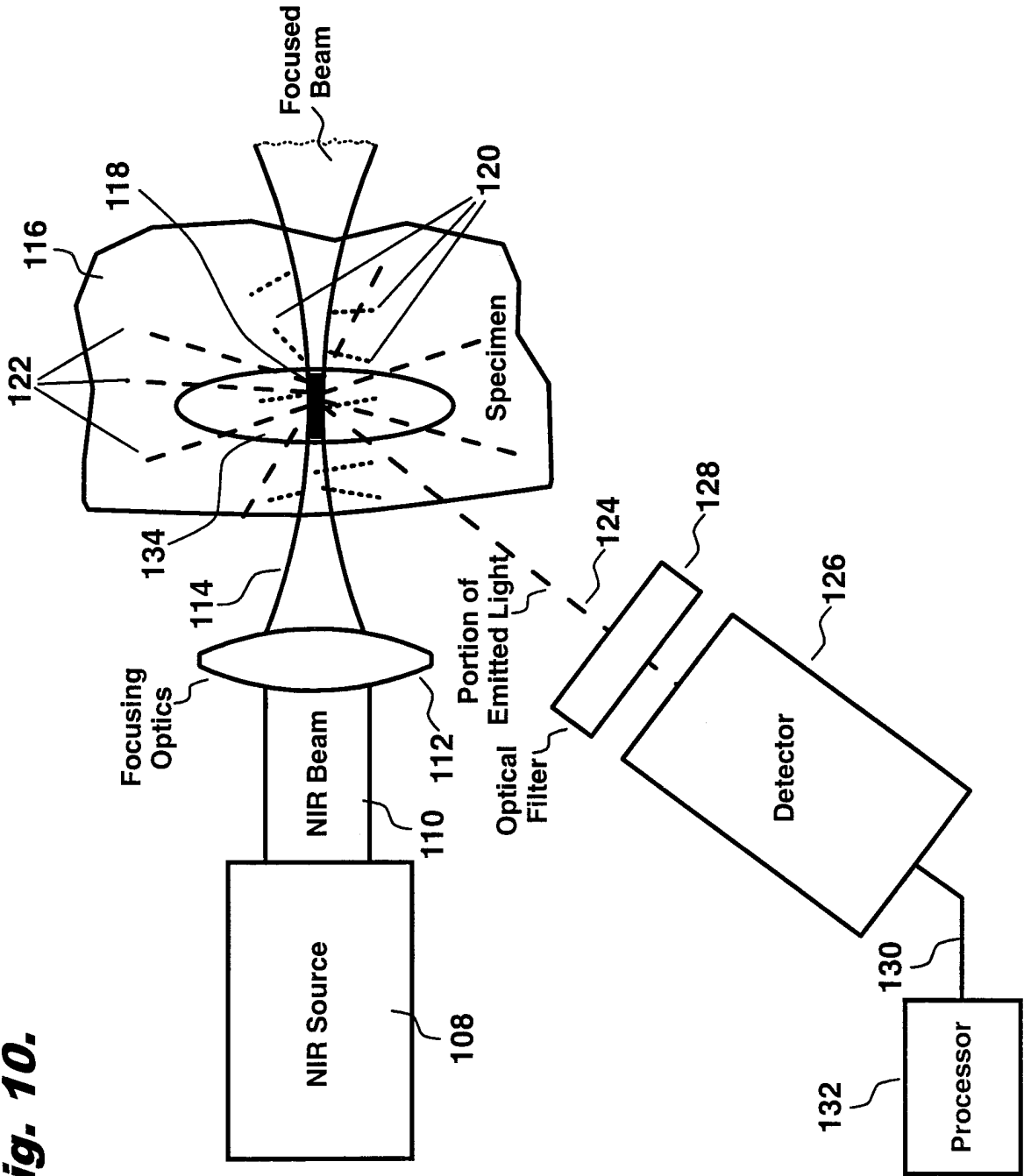


Fig. 11.

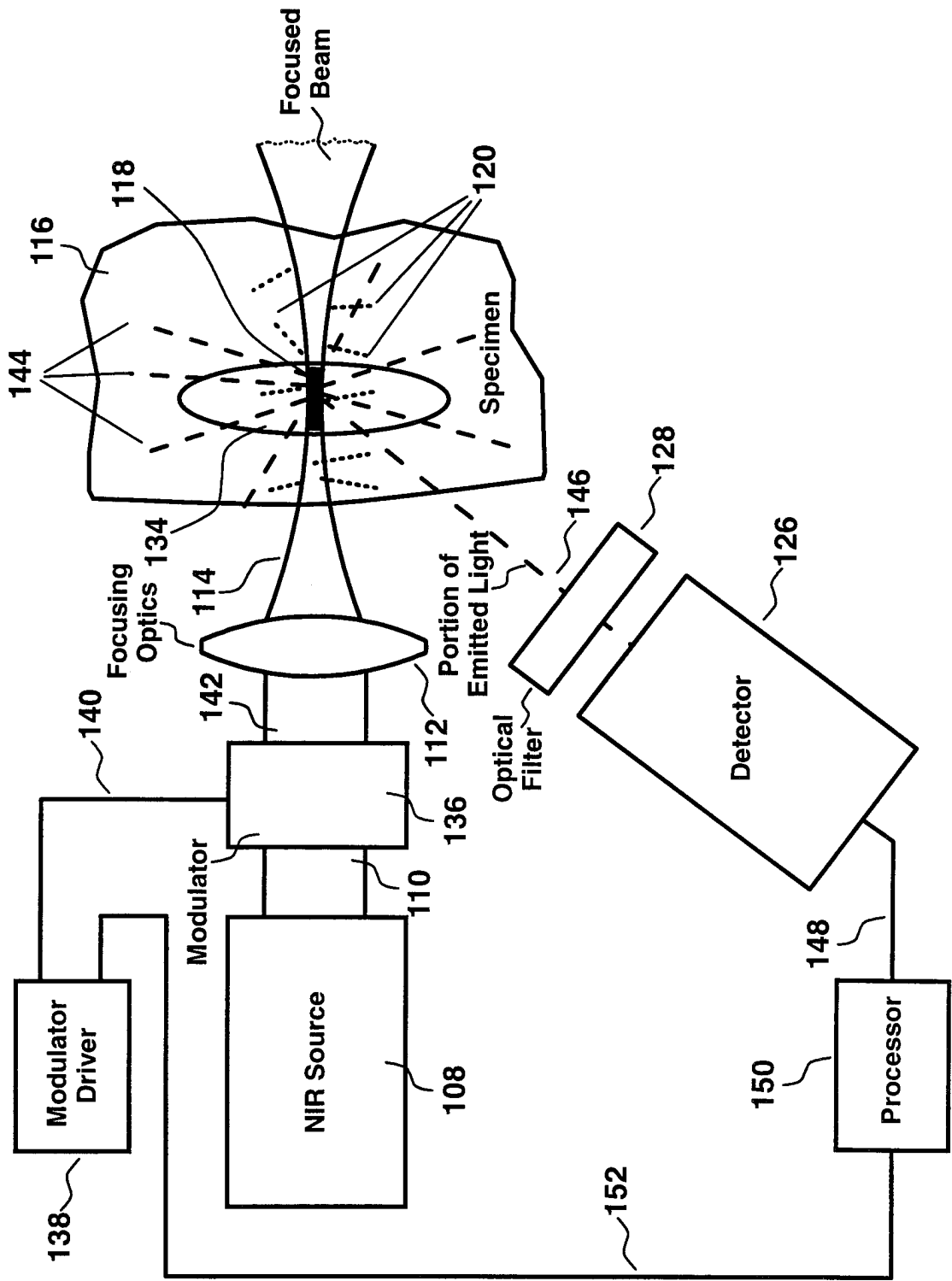
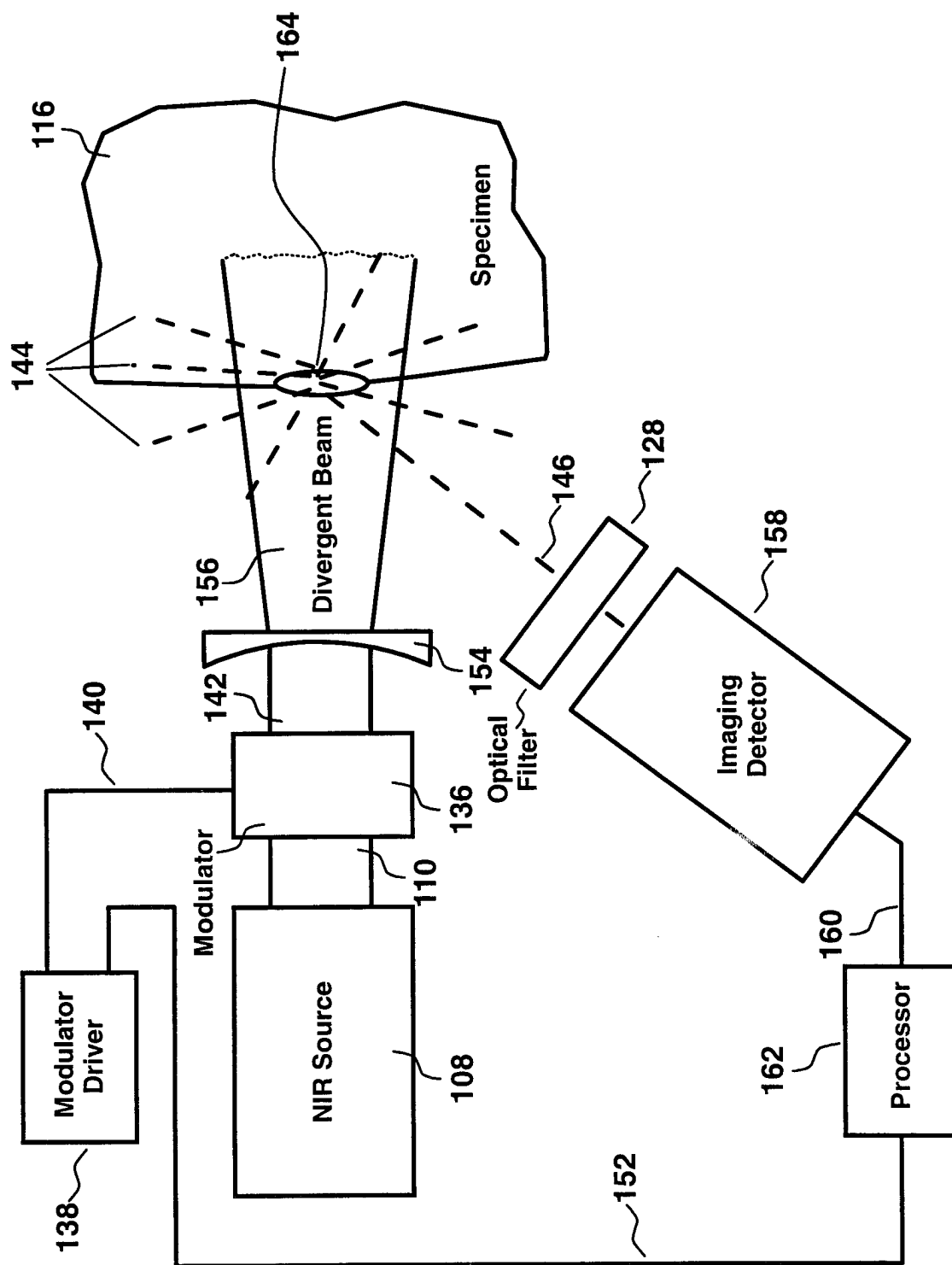


Fig. 12.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/14169

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61B 6/00

US CL :128/898; 600/300, 310, 476

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)

U.S. : 128/898; 600/300, 310, 476; 604/20; 606/9, 15, 16; 607/89

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,832,931 A (WACHTER et al.) 10 November 1998, entire document.	1-78
A,P	US 6,030,411 A (Lawandy) 29 February 2000, entire document.	1-78
A	US 5,868,731 A (BUDNIK et al.) 09 February 1999, entire document.	1-78

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier document published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

31 AUGUST 2000

Date of mailing of the international search report

13 OCT 2000

Name and mailing address of the ISA/US
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Washington, D.C. 20231

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专利名称(译)	用于多光子光活化和分子试剂检测的改进方法和装置		
公开(公告)号	EP1187555A4	公开(公告)日	2004-12-01
申请号	EP2000937691	申请日	2000-05-23
[标]申请(专利权)人(译)	致光公司		
申请(专利权)人(译)	PHOTOGEN INC.		
当前申请(专利权)人(译)	PHOTOGEN INC.		
[标]发明人	FISHER WALTER SMOLIK JOHN DEES H CRAIG WACHTER ERIC		
发明人	FISHER, WALTER SMOLIK, JOHN DEES, H, CRAIG WACHTER, ERIC		
IPC分类号	G01N21/64 A61B5/00 A61B5/103 A61B10/00 A61B6/00		
CPC分类号	A61B5/444 A61B5/0059 A61B5/0091		
优先权	60/135886 1999-05-26 US 09/569982 2000-05-10 US		
其他公开文献	EP1187555A1		
外部链接	Espacenet		

摘要(译)

一种用于诊断或成像特定体积材料的方法和设备，包括用足以促进包含在特定体积上或体内的至少一种光活性剂的多光子激发的光处理特定体积的材料的步骤所述材料，其中所述至少一种光活性剂在所述材料的特定体积中变得有活性，从而产生光学信号，并且其中以电子形式捕获所述光学信号以用于特定的特定诊断或成像。材料量。