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(54) **CONTRAST ENHANCED SPECTROSCOPIC  
OPTICAL COHERENCE TOMOGRAPHY**

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(57) **ABSTRACT**

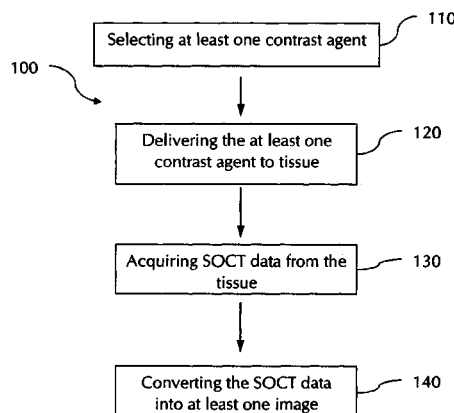
A method of forming an image of a sample includes perform-  
ing SOCT on a sample. The sample may include a contrast  
agent, which may include an absorbing agent and/or a scat-  
tering agent. A method of forming an image of tissue may  
include selecting a contrast agent, delivering the contrast  
agent to the tissue, acquiring SOCT data from the tissue, and  
converting the SOCT data into an image. The contributions to  
the SOCT data of an absorbing agent and a scattering agent in  
a sample may be quantified separately.

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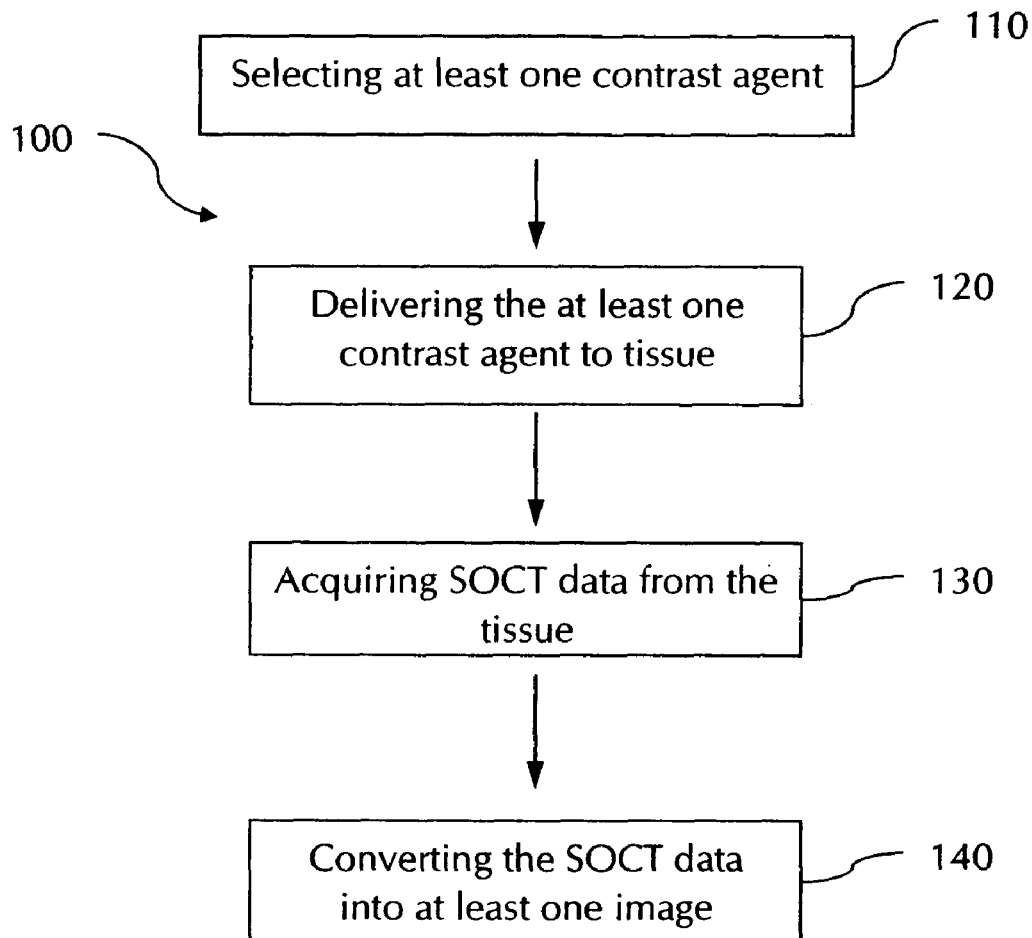
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**FIG. 1**

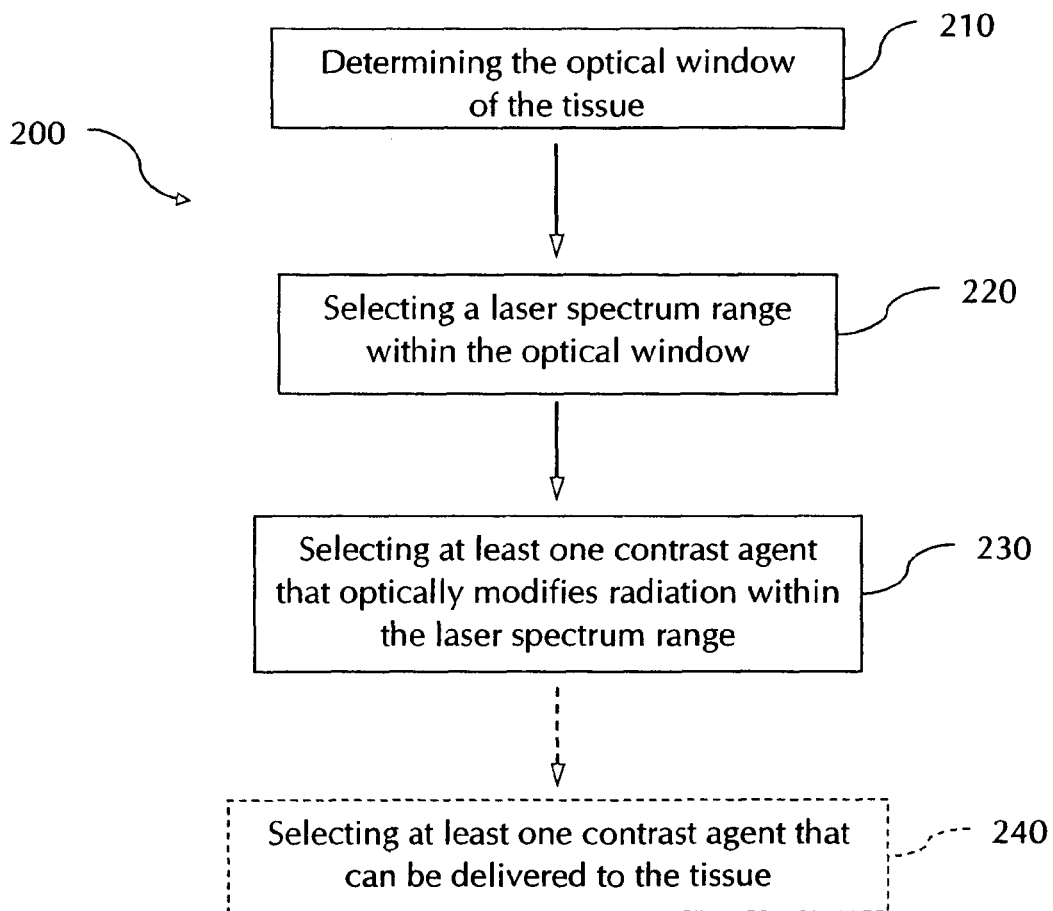


FIG. 2

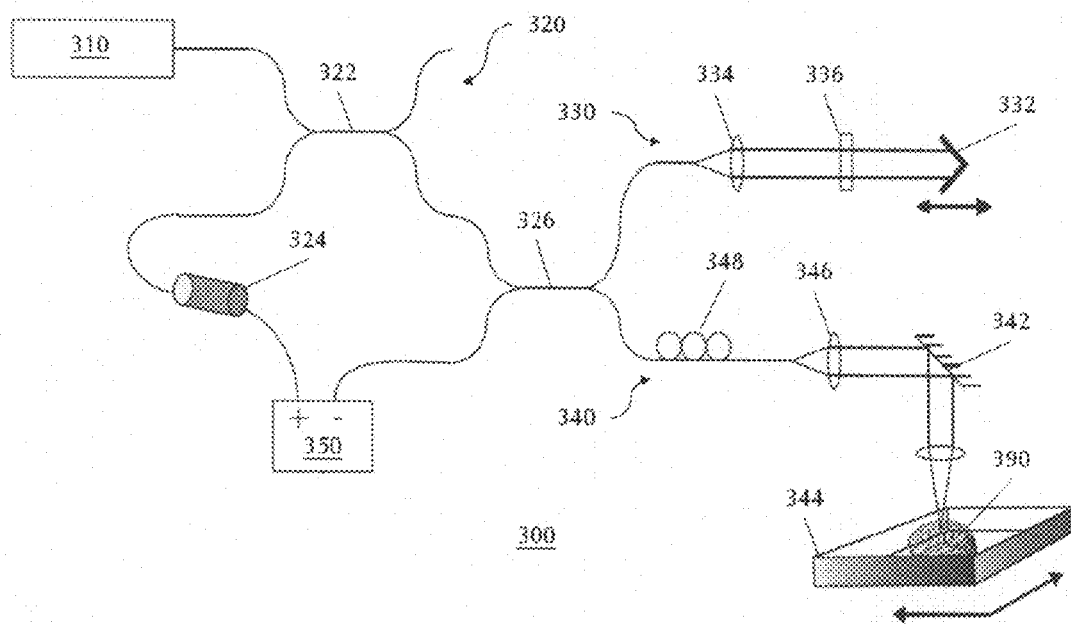
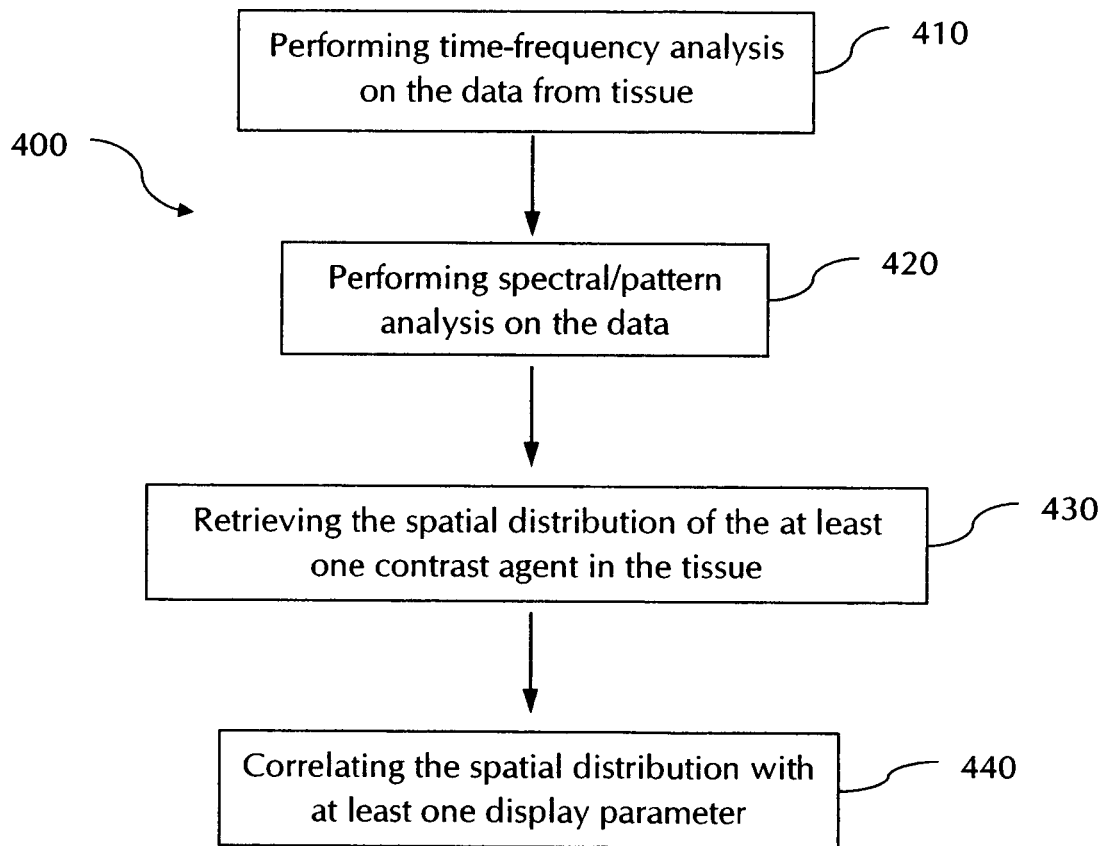


FIG. 3

**FIG. 4**

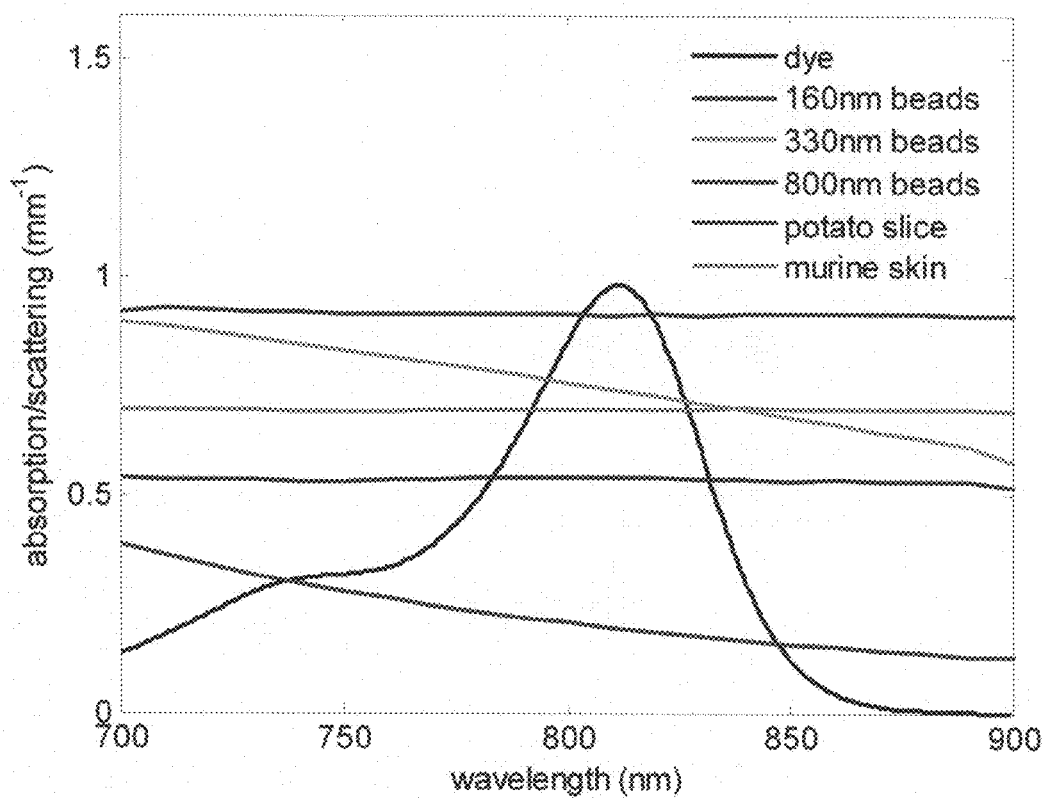


FIG. 5

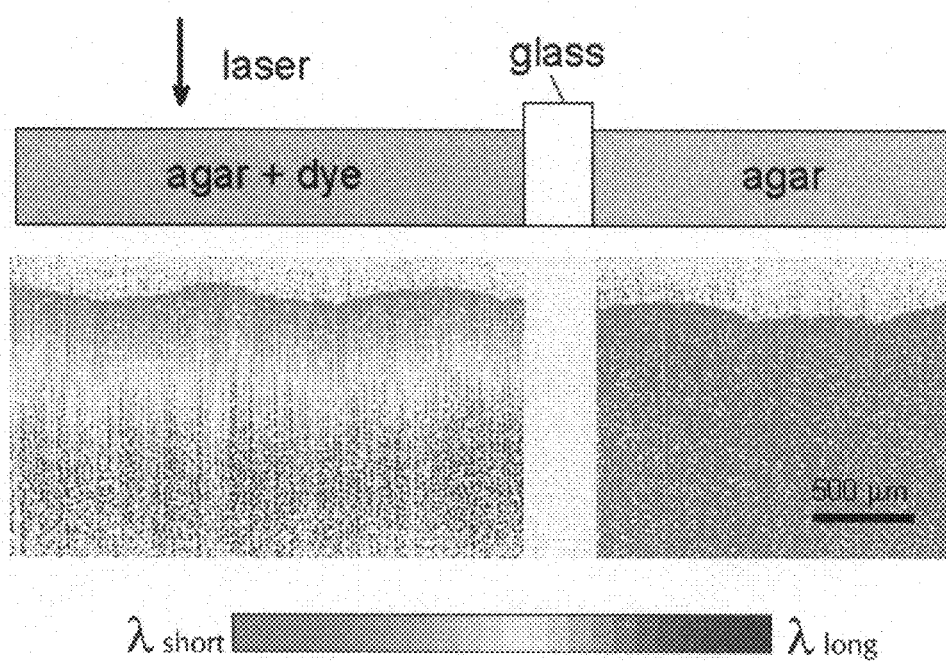


FIG. 6



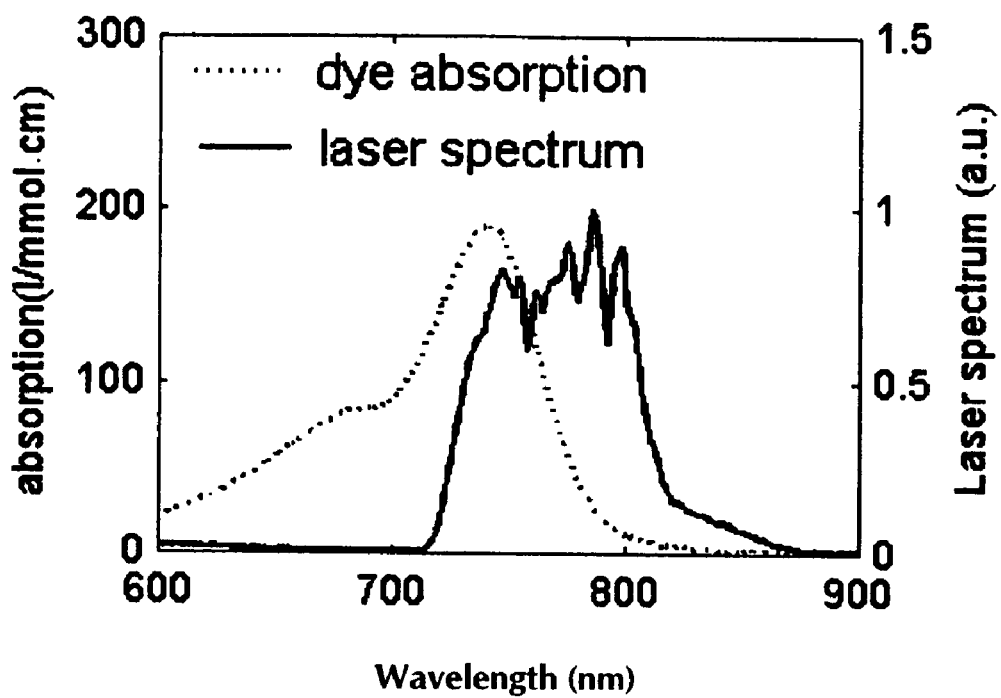


FIG. 7

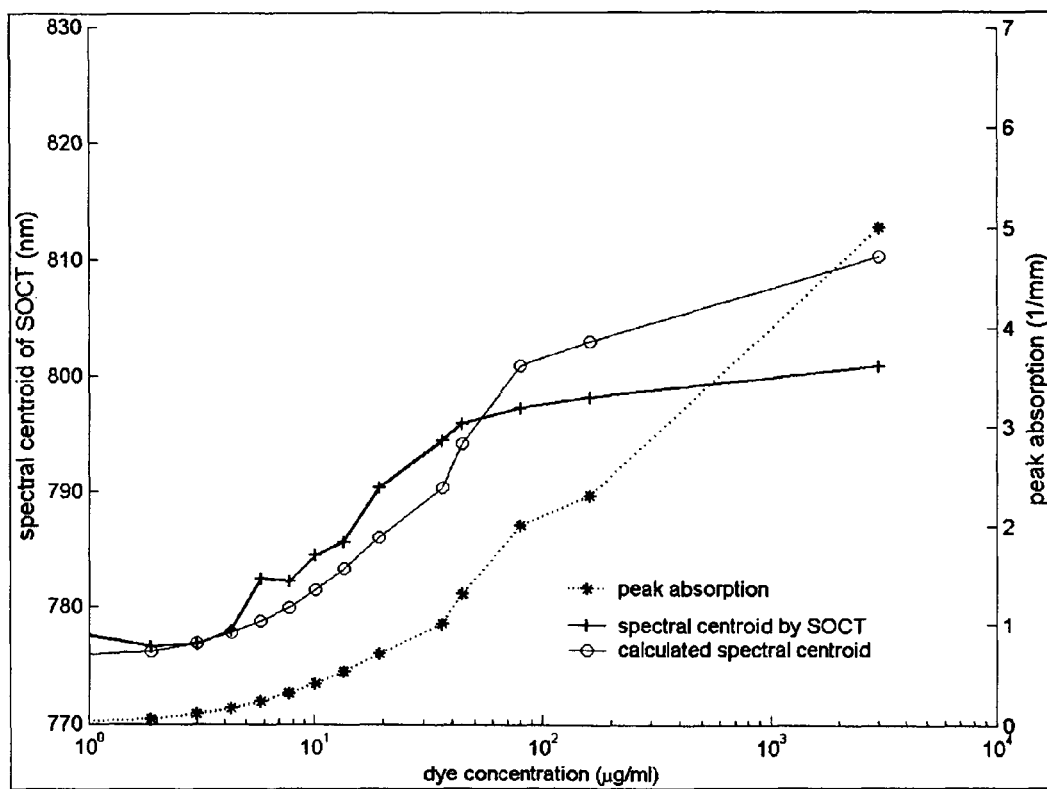


FIG. 8

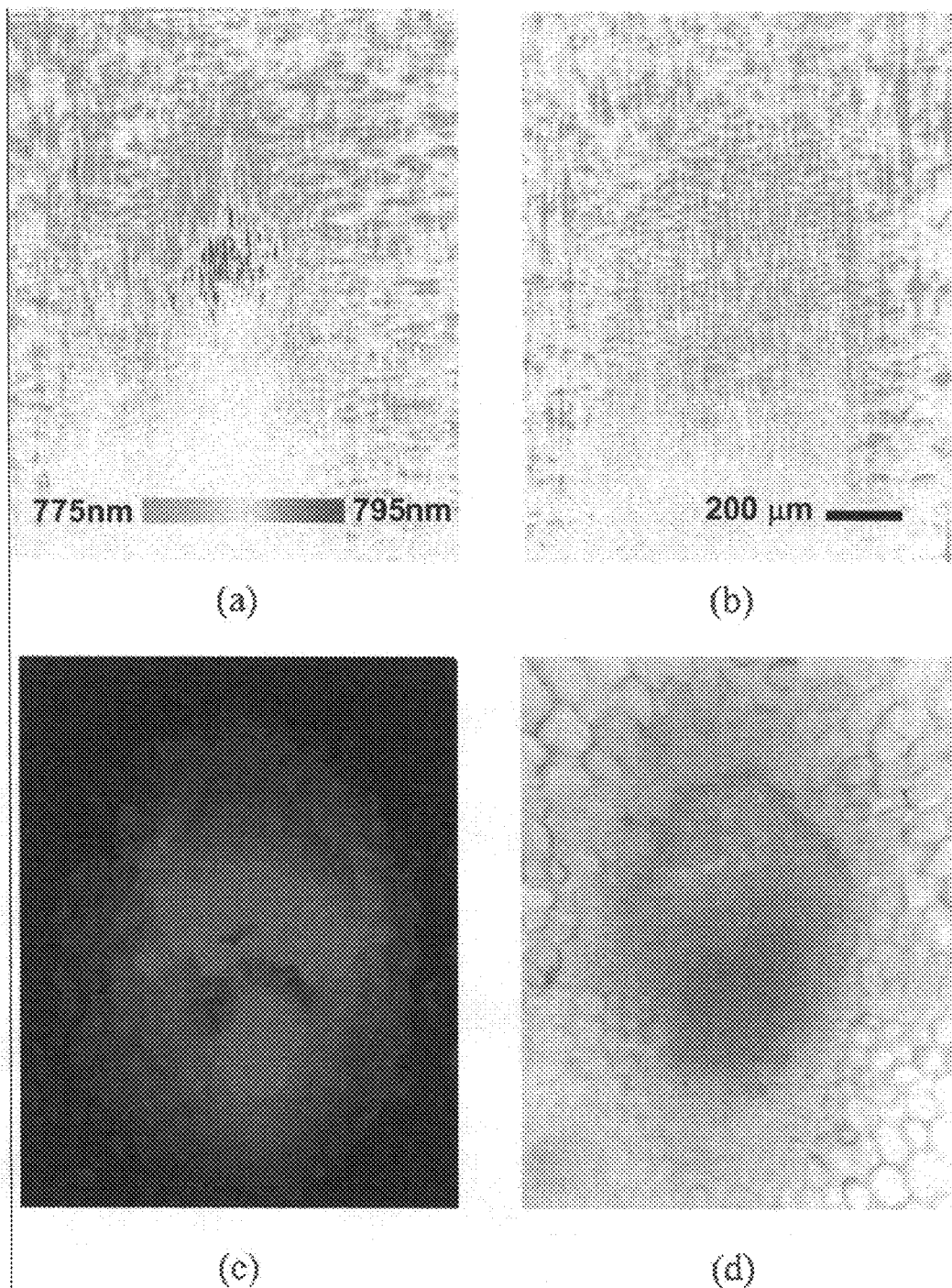


FIG. 9

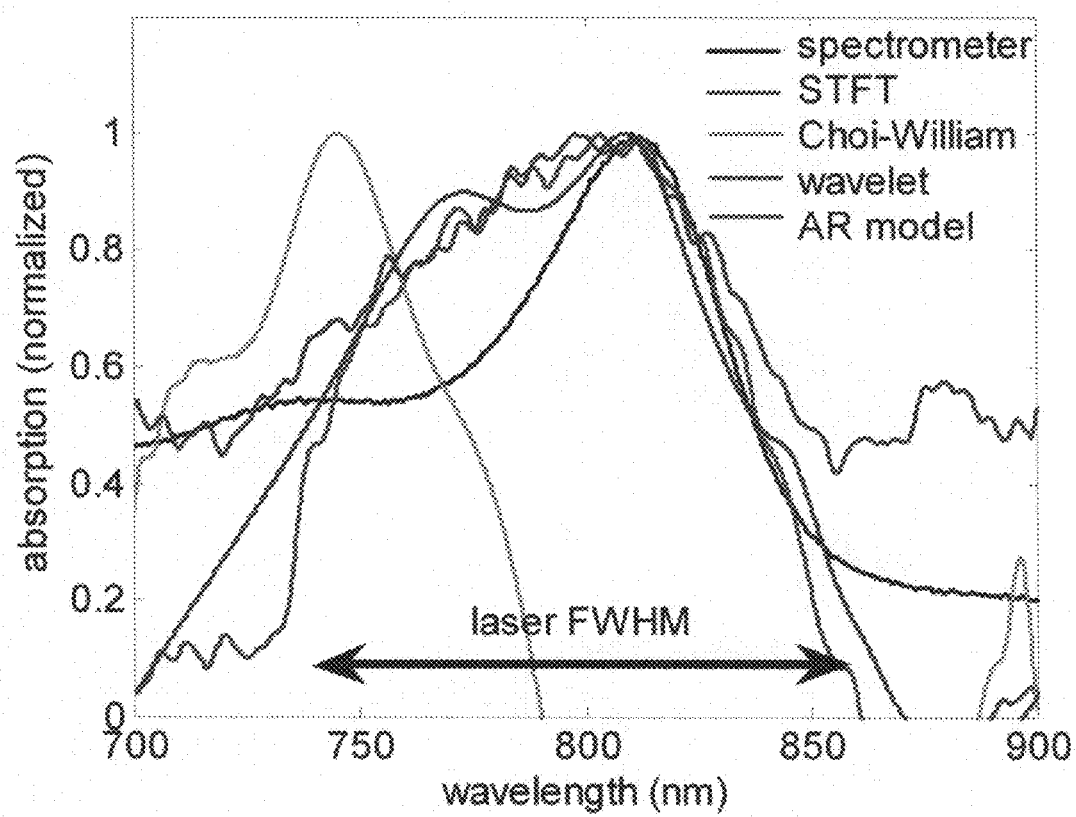


FIG. 10

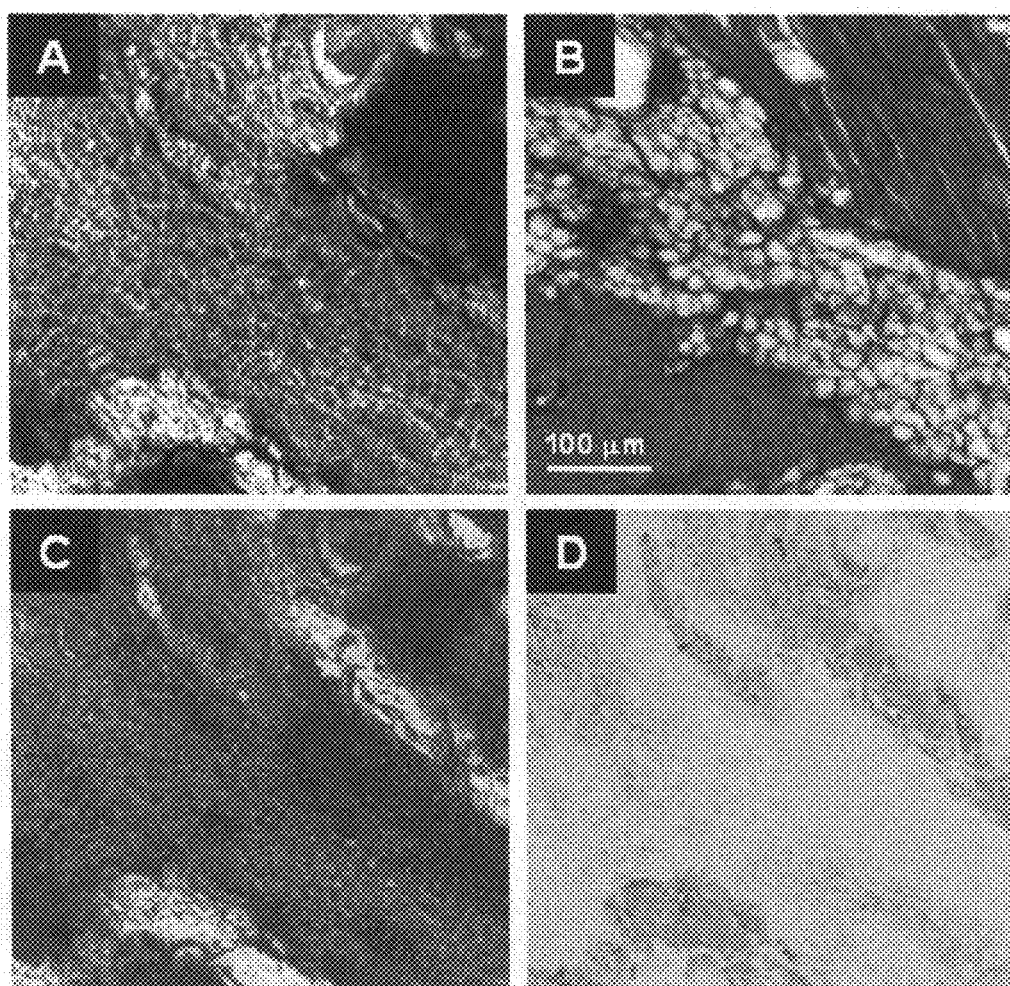


FIG. 11

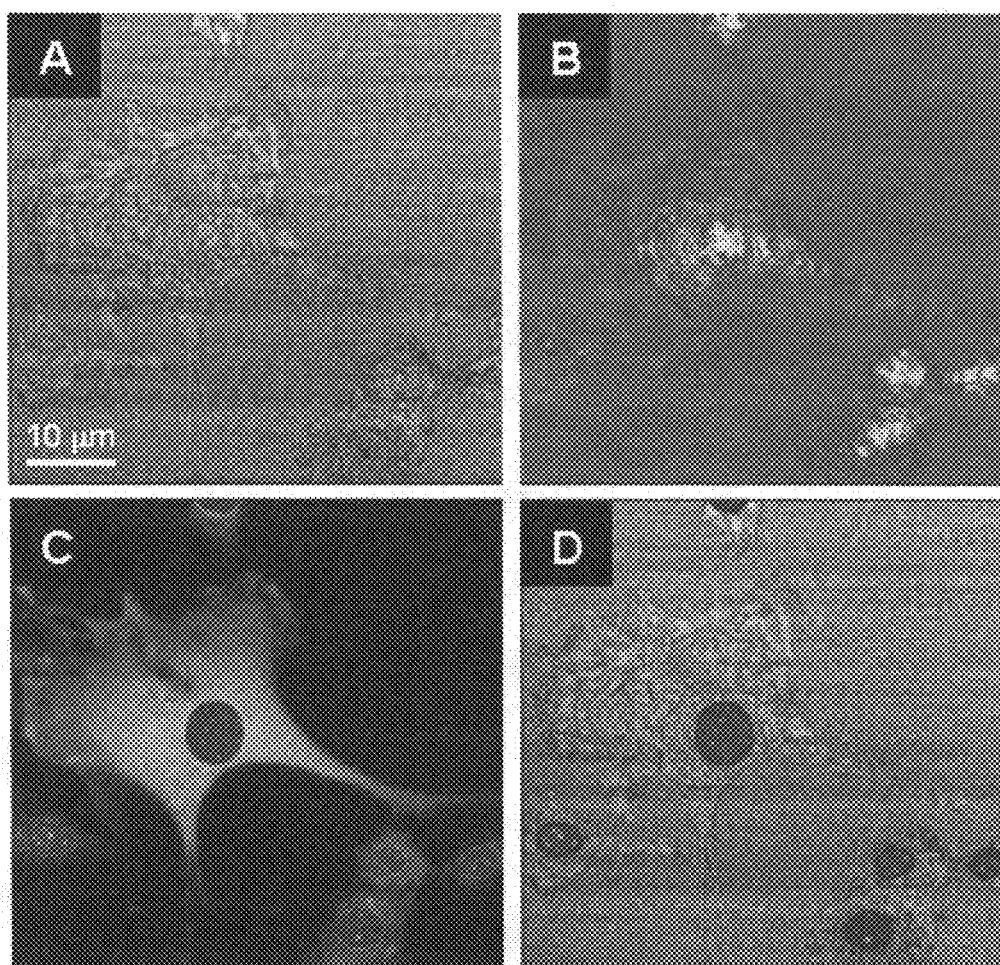


FIG. 12

# CONTRAST ENHANCED SPECTROSCOPIC OPTICAL COHERENCE TOMOGRAPHY

## REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/672,205 entitled "Contrast Enhanced Spectroscopic Optical Coherence Tomography" filed Apr. 15, 2005, which is incorporated by reference in its entirety.

## FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The subject matter of this application may have been funded in part under a research grants from the National Aeronautics and Space Administration (NASA), under Contract Number NAS2-02057. The U.S. Government may have rights in this invention.

## BACKGROUND

Optical coherence tomography (OCT) is a high-resolution medical and biological imaging technology. OCT has been used in ophthalmology for high-resolution tomographic imaging of the retina and anterior eye. Recently, the technique has been applied for imaging a wide range of nontransparent tissues to investigate applications in tissues studies and medical applications in gastroenterology, urology, and neurosurgery. OCT detects the reflections of low-coherence light, and cross-sectional imaging may be performed by measuring the backscattered intensity of light from structures in tissue. This imaging technique is attractive for medical imaging because it permits the imaging of tissue microstructure in situ. In situ imaging with OCT may provide micron-scale imaging resolution without the need for excision and histological processing.

Spectroscopic optical coherence tomography (SOCT) is an extension of OCT that can provide depth resolution and can differentiate between different types of tissue. In addition to the normal OCT measurement of the intensity of light backscattered from the sample, SOCT measures the spectral absorption and reflectance data from the tissue. Tissue structure can be resolved based on local optical densities, ignoring the frequency dependent changes. SOCT resolves both the amplitude, which contains the density information, and the frequency, which contains the spectroscopic molecular composition information.

Contrast agents may be used to improve the resolution of images obtained from an imaging technique, including OCT. Conventional contrast agents serve to increase the intensity of backscattered light. For example, air-filled micro-bubbles and engineering microspheres may be introduced into tissue to increase the back-scattering from tissue. In another example, a molecular contrast agent can be generated using a pump-probe technique to change the absorption.

A method to increase the types of tissue that may be resolved with SOCT methods would be beneficial. For example, substances such as melanin and hemoglobin exhibit strong selective absorption signature, and may be directly resolved by conventional SOCT. However, these substances are common in tissue and often may not be used to discriminate tissue types. It would be desirable to provide contrast agents that could improve and expand the application of SOCT. It would also be desirable to extract additional information from tissue samples regarding the structure and the composition of the tissue.

## SUMMARY

In one aspect, the invention provides a method of forming an image of a sample.

In another aspect, the invention provides a method of performing SOCT on a sample.

In yet another aspect, the invention provides a method of selecting a contrast agent.

In yet another aspect, the invention provides a method of selecting a combination of an absorbing agent and a scattering agent.

In yet another aspect, the invention provides a method of performing SOCT on a sample comprising at least one absorbing agent and at least one scattering agent.

In yet another aspect, the invention provides a method of enhancing the contrast of an image of a sample.

In yet another aspect, the invention provides a method of separately quantifying the contributions to SOCT data of at least one absorbing agent and at least one scattering agent in a sample.

In yet another aspect, the invention provides a method of forming an image of tissue that includes selecting at least one contrast agent, delivering the at least one contrast agent to the tissue, acquiring SOCT data from the tissue, and converting the SOCT data into at least one image.

In yet another aspect, the invention provides a method of forming an image of tissue that includes selecting at least one contrast agent, delivering the at least one contrast agent to tissue, acquiring SOCT data from the tissue, and converting the SOCT data into at least one image. The at least one contrast agent includes at least one water-soluble, biocompatible absorbing agent.

In yet another aspect, the invention provides a method of forming an image of tissue that includes determining the optical window of living tissue, selecting a laser spectrum range that is within the optical window, selecting at least one water-soluble, biocompatible absorbing agent that absorbs within the laser spectrum range, selecting at least one scattering agent that scatters within the laser spectrum range, delivering the at least one absorbing agent and the at least one scattering agent to the living tissue, acquiring SOCT data from the living tissue, performing time-frequency analysis on the data, performing spectral/pattern analysis on the data, retrieving the spatial distributions of the at least one absorbing agent and of the at least one scattering agent in the living tissue, and correlating the spatial distributions with at least one display parameter.

In yet another aspect, the invention provides a method of converting SOCT data into at least one image that includes performing time-frequency analysis on SOCT data from tissue, performing spectral/pattern analysis on the SOCT data, retrieving the spatial distribution of at least one contrast agent in the tissue, and correlating the spatial distribution with at least one display parameter.

The scope of the present invention is defined solely by the appended claims and is not affected by the statements within this summary.

## BRIEF DESCRIPTION OF THE DRAWINGS

The invention can be better understood with reference to the following drawings and description. The components in the figures are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

FIG. 1 depicts a method of forming an image of tissue.

FIG. 2 depicts a method of selecting at least one contrast agent.

FIG. 3 is a schematic representation of a device for acquiring SOCT data.

FIG. 4 depicts a method of converting SOCT data into at least one image.

FIG. 5 is a graph of scattering loss for a series of microbead solutions and tissue samples, together with a graph of absorption attenuation for an absorbing agent solution.

FIG. 6 is a schematic representation of a phantom sample and of a display of an SOCT image of the sample.

FIG. 7 is a graph of the emission spectrum of a laser, together with the absorption spectrum of an absorbing agent.

FIG. 8 is a graph of the centroid of a backreflected light spectrum as a function of absorbing agent concentration.

FIG. 9 is a series of images of the tissue of a celery stalk.

FIG. 10 is a graph of absorption spectra obtained using a variety of time-frequency distributions.

FIG. 11 is a series of images of rat mammary tissues.

FIG. 12 is a series of single-cell images of a GFP-vinculin transfected fibroblast.

### DETAILED DESCRIPTION

The present invention makes use of the discovery that contrast agents can be selected and delivered to tissue to provide for enhancement of the contrast in SOCT imaging. By the site-specific and molecule-specific introduction of absorbing agents and/or scattering agents, the invention improves OCT imaging quality, widens OCT usage areas, and provides a means of molecular imaging. The present invention also includes the use of both absorbing agents and scattering agents in the same tissue to enhance SOCT image contrast. In addition, it has been discovered that the contributions of an absorbing agent and a scattering agent to the optical properties of contrast-enhanced tissue can be quantified separately. SOCT imaging that incorporates and analyzes contrast agents according to the present invention can provide for improvements in the image quality and in the variety of tissues that can be analyzed.

The term "contrast agent" means any substance that changes the optical properties of tissue containing the substance. Optical properties that may be changed include absorbance, reflectance, fluorescence, birefringence and optical scattering.

The term "optical modification" means a change in one or more optical properties of radiation.

The phrase "contrast enhancement" means that an image produced with the enhancement shows a greater difference in optical properties between parts of the image, than an otherwise identical image produced without the enhancement.

The term "image" means data produced by receipt of electromagnetic radiation, which may or may not be formed into a picture viewable by the human eye. This includes images produced directly onto a medium such as film or video.

FIG. 1 represents a method 100 of forming an image of tissue that includes selecting at least one contrast agent 110, delivering the at least one contrast agent to tissue 120, acquiring SOCT data from the tissue 130, and converting the SOCT data into at least one image 140. The selecting at least one contrast agent 110 may include selecting at least one absorbing agent and/or may include selecting at least one scattering agent.

FIG. 2 represents a method 200 of selecting at least one contrast agent that includes determining the optical window of the tissue 210, selecting a laser spectrum range that is within the optical window 220, and selecting at least one contrast agent that optically modifies radiation within the laser spectrum range 230. If more than one contrast agent can

be selected, the method 200 optionally may include selecting at least one contrast agent that can be delivered to the tissue 240.

Determining the tissue optical window 210 may include determining a wavelength region of electromagnetic radiation that is only minimally absorbed by the tissue. When radiation having a wavelength within this optical window is passed through the tissue, attenuation of the radiation is governed by scattering rather than absorbance. Ultraviolet radiation and infrared radiation are absorbed by the majority of substances in biological systems, such as water, proteins without chromophores, carbohydrates, nucleic acids and lipids. Accordingly, the tissue optical window for most tissues is in the near-infrared (NIR) region, typically from 600 nm to 1500 nm. Analysis within the tissue optical window is preferred for deep tissue imaging.

Selecting a laser spectrum range that falls within the tissue optical window 220 may include selecting a laser source that emits radiation over a wavelength range within the optical window. Preferably the center wavelength of the radiation is within the optical window. The radiation emitted by a laser may also be filtered or frequency shifted so as to produce radiation within the tissue optical window.

Selecting at least one contrast agent 230 may include selecting at least one substance that changes one or more optical properties of tissue containing the substance when subjected to radiation within the laser spectrum range. There must be overlap between the laser spectrum range and the spectrum range in which the substance optically modifies radiation. Selecting at least one contrast agent 230 may also include selecting at least two substances, each of which optically modifies radiation passing through the tissue. Preferably each substance modifies a different property of the radiation and/or modifies radiation within a different portion of the laser spectrum range.

Selecting at least one contrast agent that can be delivered to the tissue 240 may include determining the relative biocompatibility of each of the contrast agents that optically modify radiation within the laser spectrum range. Selecting at least one contrast agent that can be delivered to the tissue 240 may also include determining which of the contrast agents that optically modify radiation within the laser spectrum range can be combined with a delivery vehicle. This selection may include considerations such as stability of the contrast agent, since the agent should exhibit useful optical modification characteristics when present in the tissue for a period of time sufficient to perform the analysis. This selection also may include considerations such as tissue specificity. Certain contrast agents may be preferentially attracted to or absorbed by different types of tissue, making these agents useful for identifying these specific tissues. Certain contrast agents may be modified to make the agents specific to certain types of tissue or to increase specificity. For example, a non-specific contrast agent may be modified with an antibody that binds to a certain type of tissue, allowing for targeting of that tissue with the contrast agent.

The contrast agent may include at least one absorbing agent. Radiation that is backscattered from tissue containing an absorbing agent will have a spectrum different from that of the impinging radiation, since a portion of the spectrum has been absorbed by the absorbing agent in the tissue. Preferably an absorbing agent has an absorption profile that is sharp, meaning that the transition region from a wavelength region that is highly absorbed to a wavelength region that is subject to little or no absorption is narrow. A sharper absorption profile typically provides for increased analytical sensitivity. Sharp absorption profiles may also be useful if multiple



absorbing agents are present, as this may provide for simultaneous analysis at different wavelengths.

Examples of absorbing agents include synthetic dyes and bio-engineered dyes. Both synthetic and bio-engineered dyes can have absorbance profiles in the near-infrared. Synthetic dyes typically are specific for small molecules and can be sensitive to pH or to concentrations of substances such as glucose, CO<sub>2</sub> and O<sub>2</sub>. These dyes can be useful for detection of tumors, since hypoxia and acidity are two well-known characteristics of tumors that grow above a certain size. Specific examples of synthetic dyes include Indocyanine Green, fluorescein, SNARF and Fura Red. Indocyanine Green (Sigma-Aldrich) typically is used for retina angiography, fluorescein (Molecular Probes) typically is used for liver function testing, SNARF (Molecular Probes) typically is used as a pH indicator, and Fura Red (Molecular Probes) typically is used as a Ca<sup>2+</sup> indicator.

Bio-engineered dyes typically are specific for proteins or for specific cellular structures. Biological basic dyes are known to preferentially stain nuclei, allowing for determination of the nuclear-to-cytoplasm ratio, which is also an important indicator of tumor progression. Other examples of bio-engineered dyes include DNA binding dyes, dye-tagged immunoproteins and natural protein chromophores. Specific examples of bio-engineered dyes include Rhodamine tagged oligonucleotide and NN382 conjugated anti-human IgG. Rhodamine tagged oligonucleotide (EMP Biotech GmbH) typically is used for DNA sequencing and blotting, and NN382 conjugated anti-human IgG (LI-COR Inc.) typically is used for protein labeling.

Examples of absorbing agents also include particles such as quantum dots, nanospheres, nanorods and nanoshells. Specific examples of absorbing particles include metal-based nanoparticles, including nanoparticles containing gold, silver, copper, cobalt, nickel, iron, and alloys or mixtures thereof. Specific examples of absorbing particles also include plasmon-resonant nanoparticles, such as those described in copending U.S. patent application Ser. No. 10/753,972 to Boppart et al., filed Jan. 8, 2004, and published as US 2005/0171433 A1. Plasmon-resonant nanoparticles include metallic nanoparticles that have an extinction coefficient of at least 10<sup>6</sup> M<sup>-1</sup> cm<sup>-1</sup> at some frequency in the infrared to ultraviolet spectrum (electromagnetic radiation in the frequency range of 10<sup>12</sup> to 10<sup>17</sup> Hz).

Examples of absorbing agents also include genetically expressed substances. For example, DsRed and hemoglobin are naturally occurring chromophores that have active near-infrared absorption and can be used as in vivo, non-invasive contrast agents. It is possible to introduce or induce over-expression of such chromophores in vivo by genetically modifying the experimental animal genome. For example, the local expression of DsRed can be achieved either by transfecting a strong promoter sequence followed by a DsRed producing gene, or by enhancing the natural DsRed producing mechanism. Examples of other genetically expressed absorbing agents include green fluorescent protein (GFP) and yellow fluorescent protein (YFP).

Absorbing agents may be encapsulated prior to delivery to the tissue. One useful aspect of encapsulation of absorbing agents is that non-biocompatible and/or water insoluble absorbing agents can be used. For example, microspheres containing absorbing agents may be constructed by encapsulating the absorbing agent in one or more layers of bovine serum protein. See, for example, copending U.S. patent application Ser. No. 10/463,833 to Suslick et al., filed Jun. 17, 2003, and published as US 2004/0258759 A1; and copending U.S. patent application Ser. No. 10/463,835 to Boppart et al.,

filed Jun. 17, 2003, and published as US 2004/0258762 A1. These microspheres may incorporate in their shells and/or in their cores a wide range of substances that can alter the local optical properties of tissue. The protein shell may also be functionalized to target agents to specific regions of interest.

The contrast agent may include at least one scattering agent in addition to the at least one absorbing agent. Examples of scattering agents include protein microspheres, microbeads and nanoparticles.

Protein microspheres have an exterior protein shell and an interior containing a gas, a liquid or particles. The compositions of the shell and the interior may be varied to produce microspheres having different spectral scattering properties. The spectral scattering properties may also be affected by the relative dimensions of the shell and the interior. See, for example, U.S. Patent Application Publication Nos. US 2004/0258759 A1 and US 2004/0258762 A1. The protein in the exterior shell can also be engineered such that melanin, gold or carbon particles are embedded. See, for example, Lee, T. M. et al., *Optics Letters*, 2003, 28(17), 1546-1548.

Simple microbeads having sizes close to the selected laser wavelength can provide Mie scattering of the laser radiation. This spectral scattering can be modified by coating the beads with dye or other materials.

Metal nanoparticle scattering agents may be solid particles or may be nanoshells. One advantage of metal nanoparticles over other scattering agents is the resistance of the nanoparticles to optical, chemical and/or thermal degradation, including denaturation and bleaching. In addition, biomolecules can be bound to nanoparticles using similar techniques to those used for gold colloids. Solid metal nanoparticles may be formed in a variety of shapes, which can affect the optical scattering properties. Metal nanoshells may have a core containing a dielectric material, and modifications in the shape, composition and relative dimensions of core and the shell can provide for systematic variation of the optical resonance over a broad wavelength region, ranging from near-UV to the mid-infrared. Gold nanoshells may be engineered to scatter or absorb light primarily in the wavelength ranges typically used for OCT.

Crystalline nanoparticles containing dielectric material may also be scattering agents. Examples of crystalline nanoparticles include Bragg reflectors and photonic crystals. Light traveling through these nanoparticles undergoes a periodic variation of the refractive index, causing a splitting of the bands at the edge of the Brillouin zone. These stop gaps appear as minima in the transmission and give rise to Bragg scattering, which is highly wavelength dependent.

Delivering the contrast agent to the tissue may be accomplished by a variety of methods. If the target tissue region is a relatively large area and can be easily accessed with a hypodermic needle, the contrast agent can be directly injected into the tissue. The contrast agent may then diffuse through tissues to create a region of high contrast. In some applications, the contrast agent can be delivered and targeted by intravenous injection. This may be useful when examining the circulatory system in tissue, when delivering the contrast agent systemically, or when the contrast agent is known to aggregate naturally in some organs or tissues. Examples of these analyses include retina angiography and analysis of liver tissue. If cells in the target tissue express specific antigens, delivery of the contrast agent may include using a contrast agent conjugated with antibodies for the antigen. For example, Cy-annexin can be conjugated with a contrast agent for tumor apoptosis studies. Examples of these modified contrast agents include commercially available targeting antibodies labeled with dyes specific for the near infrared range.

Acquiring SOCT data includes dividing low-coherence radiation between two paths, the reference path and the sample path. Radiation traveling along the reference path is reflected against a reference mirror and then collected as a reference signal. Radiation traveling along the sample path is reflected against a sample mirror and then into the sample tissue. Any radiation that is scattered back from the tissue sample is reflected against the sample mirror and then collected as a sample signal. The signals are filtered to match the dispersion and polarization and then combined into an interference pattern. The resulting interference pattern corresponds to the signal from a single point within the sample. The depth of this point is determined by the distance between the sample and the light source relative to the distance between the reference mirror and the light source, as constructive interference is maximized for signals having the same path length. Variation of these relative distances provides for signals from points at different depths within the sample. Two-dimensional in-plane translation of the sample signal relative to the sample can provide signals across a particular area of the sample.

A variety of techniques can be used to divide the laser radiation into two signals. For example, the radiation can be intersected by a partially reflective mirror, reflecting a portion of the radiation at an angle and permitting the remainder of the radiation to pass through the mirror. The radiation may also be passed through a fiber optic assembly that is configured to split the incident radiation into two fiber optic paths. Variation of the scan depth can be accomplished by moving the reference mirror and/or the sample along the path of the radiation. Variation of the lateral position of the scan can be accomplished by changing the angle of the sample mirror and/or by moving the sample.

FIG. 3 is a schematic representation of an example of a device 300 for acquiring SOCT data from a sample 390. SOCT device 300 includes a low coherence laser source 310, a fiber optic assembly 320, a reference assembly 330, a sample assembly 340 and a detector 350. The fiber optic assembly 320 may include a preliminary beam splitter 322 that diverts 10% of the radiation to adjustable attenuator 324 connected to the detector 350. The fiber optic assembly 320 includes a beam splitter 326 that divides the radiation between the reference assembly 330 and the sample assembly 340. The radiation that is reflected from the reference assembly 330 and the sample assembly 340 is directed to the detector 350. Reference assembly 330 includes reference mirror 332, which may be moved toward or away from the fiber optic assembly 320. The reference assembly 330 may include fiber collimator 334, for collection of the radiation reflected from the reference mirror 332, and may include a dispersion matching glass 336 to match the dispersion of the reference signal with the sample signal. The sample assembly 340 includes sample mirror 342, which reflects the radiation to the sample 390 in the sample holder 344. The orientation of the sample mirror 342 may be varied to provide for scanning of the radiation across an area of the sample. In addition to or instead of changes in the orientation of the sample mirror 342, the sample holder 344 may be moved along the length and width of the sample. The sample assembly 340 may include fiber collimator 346, for collection of the radiation reflected from the sample mirror 342, and may include a polarization matching paddle 348 to match the polarization of the sample signal with the reference signal. The detector 350 can perform initial processing of the signal to provide the SOCT data. Initial processing may include digitization, noise removal and digital aberration correction.

In one example of an SOCT device, the low coherence laser is a Nd:YVO<sub>4</sub> pumped titanium:sapphire source laser that has a spectrum span from 650 nm to 900 nm after passing through a non-linear fiber. Dispersion and polarization are matched in the reference and sample assemblies. A precision galvanometer is used to scan the reference mirror, and non-linearities in galvo speed are relatively small so that interferometric triggering methods are not required. Special fibers, a 3-dB splitter, lenses, signal filtering, and demodulation are used to support the broad optical and electronic bandwidths. The detector collects the full fringe data and digitizes the signal with an oversampling ratio of at least 2.

If the laser source has an ultra-broad spectrum, the imaging should be done in free space since fiber-optic components typically cannot accommodate the extremely broad spectra. A spectral domain OCT setup may also be used to improve the resolution. For applications involving real time analysis, a real time SOCT based on a field-programmable gate array (FPGA) implementation can be used. The SOCT sample radiation can be delivered to internal body locations with the use of fiber-optic probes and catheters.

FIG. 4 represents a method 400 of converting SOCT data into at least one image that includes performing time-frequency analysis on the data from tissue 410, performing spectral/pattern analysis on the data 420, retrieving the spatial distribution of the at least one contrast agent in the tissue 430, and correlating the spatial distribution with at least one display parameter 440.

Performing time-frequency (TF) analysis on the SOCT interference data 410 can provide advantages over conventional spectral analysis methods, such as the Fourier transform. Conventional methods typically are limited to use with stationary signals, whereas TF analysis offers localized spectral analysis useful for the non-stationary signals generated by SOCT. Time-frequency analysis in SOCT is described, for example, in Xu, C. et al. *Applied Optics*, 44, 1813-1822 (2005).

One aspect of TF analysis is the so-called time-frequency “uncertainty principle”, which recognizes the tradeoff between spectral resolution and time resolution. Optimization of this time-frequency resolution may be facilitated by selection of appropriate TF analysis methods, referred to as time-frequency distributions (TFDs). For the case of only one strong scatter within the coherence length, the interferometric power spectrum  $I(\omega, z)$  can be expressed as the multiplication of source spectrum  $S(\omega)$  and the modulation effect, which includes the contributions from spectral backscattering profile  $H_s(\omega)$ , the lumped spectral absorption  $H_a(\omega)$  by media before that scatter, and the total spectral modification  $H_M(\omega)$  by optical components such as beamsplitter along the optical pathways. The equation for the interferometric power spectrum  $I(\omega, z)$  is:

$$I(\omega, z) = S(\omega) H_s(\omega, z) H_a(\omega, z) H_M(\omega)$$

Usually  $S(\omega)$  and  $H_m(\omega)$  are stationary and known a priori, therefore measuring  $I(\omega, z)$  offers the opportunity to study the material properties in the sample.

TFDs for SOCT can be classified into the categories of linear TFDs, Cohen’s class TFDs and model-based TFDs. Linear TFDs are classical time-frequency analysis methods that only involve linear operations to the time domain signal. The short-time Fourier transform (STFT) and Gabor representations are the most familiar examples. The linear TFDs have the advantage that they are devoid of oscillating cross-terms, which are present for many other TFDs. Different TF tradeoffs can be made by choosing different time windows.

Linear TFDs often lead to good results, but they are compromised by the tradeoff between time and frequency resolution due to a windowing effect.

Cohen's class TFDs, also referred to as "bilinear TFDs," can be performed in many variations. One example of a Cohen's class TFD is the Wigner-Ville distribution (WVD), which can achieve better TF resolution than the linear TFDs. The main drawback with the WVD is the presence of strong cross-terms if the signal is multi-component. Cross-terms can be suppressed by using 2-D low-pass filters (kernels) in the ambiguity domain such as in the smoothed pseudo WVD (SPWVD). Another example of a Cohen's class TFD is a data-adaptive TFD that employs a radially-Gaussian kernel that is signal dependent, and thus changes shape for each signal (D. L. Jones and T. W. Parks, *IEEE Transaction on Acoustics, Speech and Signal Processing*, 38, 2127-2135 (1990)).

In model-based TFDs, the spectrum is not directly calculated. Instead, models and model parameters are estimated and used to reconstruct the spectrum. Models should be carefully chosen based on prior information. For example, if it is known that the dominating spectral modification occurring in a sample is due to the addition of a specific absorbing agent, a model can be constructed based on the laser spectrum and the absorbing agent absorption spectrum to extract the absorbing agent concentration distribution in the sample. If no prior knowledge is known, an autoregressive-moving average (ARMA) model is often used. The time localization of model-based TFDs is achieved by windowing.

The equation for the interferometric power spectrum  $I(\omega, z)$  includes terms for the contributions from the lumped spectral absorption  $H_L(\omega)$  by media before scattering and from the spectral backscattering profile  $H_S(\omega)$ . These two contributions have different requirements on the time-resolution and frequency-resolution. The spectral back-scattering is a short-range effect, in that large spectral variations can happen within a very short distance, usually between interfaces such as cell or tissue boundaries. High spatial resolution is required while spectral resolution can be somewhat relaxed because large spectral modifications are expected. In contrast, the spectral absorption or scattering loss is a relatively long-range effect following the Beer's absorption law. At typical absorber concentrations in tissue, distances larger than the coherence length of the optical source are typically required to produce significant spectral modification. Both effects may co-exist with tissue imaging.

Preferably, the TFD used to perform the TF analysis is optimized for resolving minute time-frequency variations. This optimization may include consideration of the various tradeoffs between different TFDs, the different parameter choices within TFDs, and the specific SOCT imaging application that is being considered.

For example, a comparison can be made between linear TFDs and Cohen's class TFDs. The linear TFD method STFT has a simple intuitive interpretation and by choosing windows of different lengths, different resolution tradeoffs can be made. Typically, however, one must manipulate the window depending on whether spectral variation or time variation are being estimated. For two interfaces that are very closely spaced, the STFT may be unable to resolve the components effectively. In contrast, Cohen's class TFDs typically can generate more compact TF analysis and therefore are more appropriate for imaging spectral reflections where higher time-frequency resolution is desired. However, the Cohen's-class TFDs suffer from the fact that artifacts are generated for multi-component signals. This problem may be mitigated by the fact that many kernel-based TFDs have sig-

nificantly-reduced artifact level, and that the SOCT signals are usually narrow pass-band signals corresponding only to the laser spectrum used in the experiments. Frequently, the artifacts from TFDs are out of the pass-band and can therefore easily be removed by filtering.

Continuing this comparison of STFT and Cohen's class TFDs, the increase in joint time-frequency resolution offered by Cohen's-class TFDs is not necessarily optimal in all SOCT imaging applications. When imaging tissue absorption or when using low-concentrations of absorbing agents as contrast-enhancing agents, significant absorption frequently requires a long pathlength. For this case, even the STFT, with its lower spatial resolution, can be sufficient. Because the STFT is totally devoid of artifacts, this TFD is the most reliable for such applications. In addition, computing STFT is significantly faster than other TFDs because of the use of the fast Fourier transform (FFT). The flexibility of digital processing permits essentially arbitrary transformation. One could potentially run a fast and less accurate STFT first, identify the potential absorbing and spectrally-reflecting locations, and then run different TFDs in the desired regions to obtain the best information. When the scattering agents are very close together and comparable to the coherence length, usual spectral analysis methods may not be reliable, as it may not be accurate to assume that the frequency components shown on the TFD plots are actually the frequency components representative of that particular spatial point. Instead, pattern analysis algorithms are better suited for identifying different objects. Digital signal processing algorithms applied to experimentally-acquired SOCT data may provide advantages in extracting diagnostic and quantitative information.

Performing spectral/pattern analysis on the SOCT interference data **420** can separate the signal due to the absorbing agents from the signal due to the scattering agents. Spectral analysis is based on the difference between the individual spectral features of the contrast agents and the individual spectral features of the endogenous material. Pattern analysis is based on the overall spectral profiles of the contrast agents. For example, the absorbing and/or scattering spectrum of a contrast agent may have a unique combination (i.e. "pattern") of modulations and features that can be matched against known optical profiles. Both spectral analysis and pattern analysis can provide for extraction of useful data regarding the presence and location of the contrast agents within tissue.

In one example of spectral/pattern analysis, the SOCT data examined is from a relatively homogenous tissue sample containing an absorbing agent having a known spectrum. This type of spectral/pattern analysis is described, for example, in Xu, C. et al. *Opt. Express* 12, 4790 (2004). The homogeneity provides for scattering spectra of endogenous scattering agents that are similar among different tissue layers. The scattering profile of endogenous scatterers in this case is mostly a linear function. For example, FIG. 5 is a graph of scattering loss as measured by a spectrometer for a series of microbead solutions (1% solution of 160 nm silica microbeads; 0.5% solution of 330 nm silica microbeads; and 0.5% solution of 800 nm silica microbeads) and for tissue samples of a thin potato slice and of murine skin. This graph also includes the absorption attenuation of a 40  $\mu$ M solution of the near-infrared dye ADS830WS (American Dye Sources, Inc.). All specimens examined showed that scattering loss was linearly dependent on wavelength, with correlation coefficients ranging from 0.987 to 0.999. In comparison, the correlation coefficient for the absorbing agent absorption profile was measured at 0.202. This spectral difference can be used to

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separate the contribution of the absorbing agent from the contribution of the scattering agent.

One possible method for separation of the absorbing agent from the scattering agent in this spectral/pattern analysis is the "least-squares fitting" algorithm. If the wavelength dependent factors are eliminated from the interferometric power spectrum  $I(\omega, z)$  equation, the interference signal may be expressed as:

$$I'(\lambda, z) \triangleq I(\lambda, z) / I(\lambda, z=0) \\ = R'(\lambda) \exp\left\{-2 \int_0^z [\mu_a(\lambda, z') + \mu_s(\lambda, z')] dz'\right\}$$

Because the same NIR dye of high absorptivity is used, the absorption coefficient at certain depths depends only on the absorbing agent concentration present at that depth. Assuming the scattering agents have similar spectral loss along sample depth, for the first-order approximation, both  $\mu_a(\lambda, z)$  and  $\mu_s(\lambda, z)$  are separable functions in  $\lambda$  and  $z$ :

$$\mu_a(\lambda, z) = \epsilon_a(\lambda) f_a(z)$$

$$\mu_s(\lambda, z) = \epsilon_s(\lambda) f_s(z)$$

where  $f_a(z)$  represents the absorbing agent concentration and  $f_s(z)$  represents the scattering agent concentration at a particular depth  $z$ . The functions  $\epsilon_a(\lambda)$  and  $\epsilon_s(\lambda)$  represent the absorption and scattering per unit concentration and per unit pathlength, and can be measured by a laboratory spectrometer or by integrating spheres. Substitution of these  $\epsilon(\lambda)$  expressions into the interference signal expression, followed by taking the logarithm of both sides yields:

$$Y(\lambda, z) \triangleq \log[I'(\lambda, z)] \\ = \log R'(\lambda) - 2 \left[ \epsilon_a(\lambda) \int_0^z f_a(z') dz' + \epsilon_s(\lambda) \int_0^z f_s(z') dz' \right] \\ = -\epsilon_a(\lambda) F_a(z) - \epsilon_s(\lambda) F_s(z) + C(z).$$

Thus,  $F_a(z)$ ,  $F_s(z)$ , and  $C(z)$  are wavelength independent functions to be found. The values of  $Y(\lambda, z)$  may be obtained by time-frequency analysis of the SOCT data.

The  $Y(\lambda, z)$  equation typically can only be solved with some optimality criteria due to the presence of noise and other non-ideal conditions. In one example, weighted minimal-mean-square-error (MMSE) optimization provides an unbiased optimization and has minimum-variance properties. After application of an estimation error variable using a weighting function that emphasizes the more accurate data, the least squares solution is:

$$\begin{bmatrix} \int_{\lambda_1}^{\lambda_2} \epsilon_a^2(\lambda) W(\lambda) d\lambda & \int_{\lambda_1}^{\lambda_2} \epsilon_s(\lambda) \epsilon_a(\lambda) W(\lambda) d\lambda & \int_{\lambda_1}^{\lambda_2} \epsilon_a(\lambda) W(\lambda) d\lambda \\ \int_{\lambda_1}^{\lambda_2} \epsilon_a(\lambda) \epsilon_s(\lambda) d\lambda & \int_{\lambda_1}^{\lambda_2} \epsilon_s^2(\lambda) W(\lambda) d\lambda & \int_{\lambda_1}^{\lambda_2} \epsilon_s(\lambda) E(\lambda) d\lambda \\ \int_{\lambda_1}^{\lambda_2} \epsilon_s(\lambda) W(\lambda) d\lambda & \int_{\lambda_1}^{\lambda_2} \epsilon_s(\lambda) W(\lambda) d\lambda & \int_{\lambda_1}^{\lambda_2} W(\lambda) d\lambda \end{bmatrix} \begin{bmatrix} F_a(z) \\ F_s(z) \\ C(z) \end{bmatrix} = \begin{bmatrix} -Y(\lambda, z) \epsilon_a(\lambda) \\ -Y(\lambda, z) \epsilon_s(\lambda) \\ -Y(\lambda, z) \end{bmatrix}$$

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-continued

$$\begin{bmatrix} -F_a(z) \\ -F_s(z) \\ C(z) \end{bmatrix} = \frac{1}{X}$$

$$\begin{bmatrix} \int_{\lambda_1}^{\lambda_2} Y(\lambda, z) \epsilon_a(\lambda) W(\lambda) d\lambda \\ \int_{\lambda_1}^{\lambda_2} Y(\lambda, z) \epsilon_s(\lambda) W(\lambda) d\lambda \\ \int_{\lambda_1}^{\lambda_2} Y(\lambda, z) W(\lambda) d\lambda \end{bmatrix}$$

Matrix  $Y$  is obtained from the SOCT measurements. System matrix  $A$  is independent of depth  $z$ . Once  $F_a(z)$ ,  $F_s(z)$ , and  $C(z)$  of matrix  $X$  are solved, the absorbing agent concentration profile  $f_a(z)$  and the scattering agent profile  $f_s(z)$  can be solved using the definitions:

$$F_a(z) \triangleq 2 \int_0^z f_a(z') dz',$$

$$F_s(z) \triangleq 2 \int_0^z f_s(z') dz',$$

$$C(z) \triangleq \log R'(\lambda, z).$$

There are typically two experimental scenarios in SOCT. The first scenario involves structures that have distinctive layers, such as experiments with cuvettes or layered phantoms. For this scenario, the parameters for time-frequency analysis may be chosen for less time resolution but higher spectral resolution. Far-spaced distinctive  $z$  points may be taken, and the least squares matrix can be solved. The second scenario involves structures that do not have distinctive layers, such as biological tissues or inhomogeneous phantoms without apparent layering. For this scenario, appropriate time-frequency analysis should be chosen with the parameters optimized to meet specific needs. Cumulative absorption  $F_a(z)$  and scattering  $F_s(z)$  may be calculated, providing for retrieval of the absorbing agent profile  $f_a(z)$  and scattering agent profile  $f_s(z)$ .

In another example of spectral/pattern analysis, the SOCT data examined is from a tissue sample containing multiple scatterers, which may include endogenous scatterers and exogenous contrast agents. The SOCT analysis may be combined with analysis methods from light scattering spectroscopy (LSS). This type of spectral/pattern analysis is described, for example, in Xu, C. et al. *Opt. Express* 13, 5450 (2005), and in Xu et al. *Opt. Lett.* 31, 1079, (2006). The imaging volume represented by a voxel in a standard OCT image is defined by the Gaussian beam width and the coherence gating, centered at the nominal voxel position. The voxel intensity is a coherent sum of scattering from all scatterers inside the imaging volume. In SOCT, due to the time-frequency uncertainty principle, in order to achieve reasonable spectral resolution, the imaging volume is usually considerably larger than in standard OCT. The imaging volume in SOCT is defined by the Gaussian beam width and the coherence gating of a particular spectral sub-band (or the time window length if the STFT is used). Although the imaging

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volume in SOCT is larger than in standard OCT, the single scattering approximation still holds for most cases.

Assuming all single-scattering events, the collected OCT signal intensity from N scatterers inside an imaging volume in the spectral domain is

$$|I(k_0)| = |C(k_0)| \left| H(k_0) * \sum_{n=1}^N S_n(k_0, P_n) \right|$$

where H is the Fourier transform of the time window, described as a spatially dependent function  $h<z>$ . The function  $S(k_0, P)$  represents the field coupled back into the lens, and is the sample-arm field for the OCT interferometer. Calculating this by integrating the secondary sources over the collection beam profile, and simplifying due to the use of the same set of optics for illumination and collection, provides

$$\sum_{n=1}^N S_n(k_0, P_n) = \sum_{n=1}^N \iint C(k_0) F_i(q_i) F_i^*(q_a) e^{i[r_0(k_i - k_a)]} R(k_i, k_a, k_0, P_n) d^2 k_i d^2 k_s$$

where  $R(k_i, k_s, k_0, P_n)$  represents the wavelength-dependent scattering amplitude of the n-th scatterer located at the origin.

These equations indicate that the scattering-mode SOCT signal may be obtained from a coherent superposition of the fields scattered from many plane waves and by many scatterers. In standard LSS, particle size is determined by observing the spectrum of the scattered field and matching the spectral signature to a particular particle size. The spectral interference arising from the coherent superposition may make such a procedure complicated for scattering-mode SOCT, since the measured OCT spectral intensity typically has a modulation term that depends on the number and the positions of the scatterers. Algorithms may be used to jointly estimate the scatterer property and location. In one example, when it is known a priori that the sample consists of practically identical particles, many incoherent SOCT measurements may be averaged. Conventional LSS may perform this incoherent averaging by using a large beam width and spatially-incoherent light sources. In a second example, an over-sampling procedure may allow an accurate estimation of particle size when one expects only one large scatterer surrounded by many smaller scatterers within the SOCT voxel.

Scatterers may be sized based on the measured spectra. For example, one approach is based on pitch detection such as using the Fourier transform or determining the autocorrelation. The principle behind this approach is that the oscillation “frequency” in the wavelength-dependent scattering is size dependent, such that larger scatterers tend to produce more oscillatory patterns. A second approach is based on curve fitting such as using least-square or  $c^2$  methods. This second approach provides an exhaustive search of possible scattering sizes and attempts to fit the normalized experimental measurement to the theoretical prediction.

In many cases, tissue demonstrates layered or regional structure where adjacent scatterers (either in axial or transverse directions) are more or less homogeneous. If weak focusing (low NA) is used, the actual single-scatterer spectral scattering may be resolved by extensive incoherent averaging.

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ing. Although OCT is typically referred to as a coherent high-spatial resolution imaging method, there are several occasions when incoherent averaging is possible over adjacent scan lines. Incoherent averaging is also possible by utilizing many so-called “diversity” methods used in OCT speckle-reduction, such as polarization or angular diversity.

Perhaps the most common SOCT scenario in biological imaging is that of one large scatterer surrounded by several small scatterers. For example, cells may have only one nucleus, but may have several mitochondria and multiple other small scatterers. It is often desirable to resolve the wavelength-dependent scattering due to the large scatterer in the presence of these smaller scatterers. For many cases, the spectrum measured by SOCT in this scenario depends on the exact location of the large scatterer within the imaging beam. If the large scatterer is in the center of the beam, the scattering is dominated by the larger scatterer. When the large scatterer is gradually moved off-center from the central region of the Gaussian beam, the scattering profile for the large scatterer is gradually corrupted by the modulation effect due to the presence of the small scatterers. This means that in some cases, the scattering due to the large scatterer can be resolved by over-sampling the SOCT signal while transverse scanning, followed by a computational search for the signal maximum.

Retrieving the spatial distribution of the at least one contrast agent in the tissue **430** includes selecting a parameter that correlates with the value of the absorbing agent profile or the scattering agent profile at a given point, followed by quantifying that parameter. Examples of quantifiable parameters include the spectral centroid shift, the Beer’s law determination of spatial distribution, and correlation strength with expected spectra. These spatial distributions may be retrieved for some or all of the scanned positions within the tissue.

The spectral centroid of SOCT interference data that has been analyzed by TFD is expressed as:

$$Centroid(z) = \frac{\int_0^\infty \omega gTFD(\omega, z) d\omega}{\int_0^\infty TFD(\omega, z) d\omega}$$

The spectral centroid location and its shifting property can be used as a parameter to characterize the contrast agent distribution. For example, if there is a particular absorbing agent in one location that absorbs preferentially low frequency light, then the spectral centroid determined by the TFD of the data from that location will be shifted toward higher frequency.

The Beer’s law determination of concentration can be used, assuming that the system can be regarded as a purely absorbing region that follows Beer’s law. The magnitude of  $H(\omega)$  expressed in wavelength can be written as a function of the depth  $z$  in the sample:

$$H(\lambda, z) = \exp \left[ - \int_{z_1}^{z_2} 2z \cdot \mu(\lambda, z) dz \right]$$

Assuming the spatial analysis window is from  $z_i$  to  $z_{i+1}$ , then:

$$\int_{z_i}^{z_{i+1}} \alpha(\omega, z') dz' = \frac{1}{2} \ln \frac{|H_i(\omega, z)|}{|H_{i+1}(\omega, z)|}$$

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Solving this integral equation by stepwise approximation, provides  $\alpha(\omega, z)dz$ , which is a quantifiable parameter.

Correlating the spatial distribution with at least one display parameter 440 includes assigning the values measured by the SOCT data to parameters that can be combined into a single display. For example, the two sets of values to be displayed may be the backscattering intensity, determined by the scattering agent profile  $f_s(z)$ , and the spatial distribution of the contrast agent. These values may then be plotted in two-dimensions, corresponding to a plane within the tissue.

In one example, an agar phantom sample containing an absorbing agent on one side and no absorbing agent on the other side was imaged by SOCT. FIG. 6 is a schematic representation of the sample, together with a color display of the SOCT image. A hue-saturation-luminance color space was used to map the backscattering intensity  $I(x, z)$  into the saturation parameter and to map the absorbing agent spatial distribution into the hue, keeping the luminance constant. This approach permitted the intensity and the spectral parameters of the backscattered light to be visualized in a 2D map. When absorbing agent is present, and with increasing depth, the short wavelengths components of the backscattered spectrum are more strongly absorbed, giving a red-shift hue for greater depths. When the absorbing agent is not present, no significant change is found.

The following examples are provided to illustrate one or more preferred embodiments of the invention. Numerous variations can be made to the following examples that lie within the scope of the invention.

## EXAMPLES

## Example 1

## SOCT Device

A fiber-based OCT setup was used for these studies. A diode-pumped mode-locked titanium:sapphire laser source with a center wavelength around 780 nm was used as the optical source. This laser pumped an ultrahigh numerical aperture (UHNA4, Nufern) fiber to spectrally broaden the output bandwidth to 120 nm. Dispersion and polarization were matched in the interferometer arms. A precision linear optical scanner was used to scan the reference arm, and the small nonlinearity (less than 0.5%) was corrected by calibration. The axial resolution of this system was measured to be 3  $\mu\text{m}$  in air. A high-speed (5 Mega-samples per second, 12-bit) analog-digital converter (NI-PCI-6110, National Instruments) was used to acquire interferometric fringe data. Axial scans containing the interferometric signals were sampled at 100,000 data points, and at 512 transverse positions to form two-dimensional images.

The collected data were analyzed using Matlab for envelope detection and depth-resolved spectroscopic information. Time-frequency analysis was performed using the short-time Fourier transform (STFT). For experiments in which high depth resolution was not required, a STFT window size of 16,384 points (corresponding to a length of 327  $\mu\text{m}$  in air) was chosen to allow for spectral resolution of 1 nm. For experiments in which both spectral resolution and depth resolution were required, the STFT window size was chosen to optimize the time-frequency concentration, typically using 1024 points (20  $\mu\text{m}$  in air). To increase the signal-to-noise ratio when recovering the absorbing agent absorption spectrum, the absorption spectra calculated was averaged over 512 measurements in cases in which lateral resolution was not required.

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## Example 2

## Absorbing Agent Selection

As noted in Example 1, a diode-pumped mode-locked titanium:sapphire laser source was selected as the optical source, due to its center wavelength around 780 nm. This wavelength, and the 120 nm window of the ultrahigh numerical aperture fiber, provided for imaging of tissue due to the transparency of most tissue components in this wavelength window.

A near-IR (NIR) absorbing agent was selected based on the spectrum of the optical source. The NIR dye ADS7460 (H.W. Sands, Inc.) has a sharp peak at 740 nm. FIG. 7 is a graph of the emission spectrum of the laser (solid line) compared to the absorption spectrum of the absorbing agent (dashed line). As shown in FIG. 7, the absorbing agent, when used in appropriate concentrations, absorbed the shorter half of the laser spectrum wavelengths and transmitted the longer half, producing a predictable spectral signature. This absorbing agent could also be encapsulated within protein microspheres, which could be used as delivery vehicles. For example, the absorbing agent could be encapsulated within bovine serum albumin microspheres.

## Example 3

## Characterization of Absorbing Agent

Various concentrations of absorbing agent solution containing the dye of Example 2 were prepared. These solutions were placed in 1-mm thick glass cuvettes (QS-459, Nova Biotech) and imaged with SOCT. The interference data from light scattered back from the top and bottom absorbing agent-glass interfaces were recorded and analyzed to extract the spectra. The absorption spectrum of the absorbing agent solution was obtained using Beer's Law, as outlined by Faber et al (D. J. Faber, E. G. Mik, M. C. G. Aalders, and T. G. van Leeuwen, *Opt. Lett.* 28, 1436 (2003)). The centroid of the backreflected light spectrum was calculated in order to display the spectroscopic data in a color image.

FIG. 8 is a graph of the centroid of the backreflected light spectrum as a function of the absorbing agent concentration. The dotted curve shows the peak absorption at 740 nm measured by a spectrometer. The shift of the spectral centroid increased and then reached a plateau with increasing absorbing agent concentration. The increase corresponded well to the theoretical calculation based on absorption data. Because OCT typically has penetration depths of 1-2 mm, an absorbing agent concentration of 50  $\mu\text{g/mL}$  could produce the largest usable shift within this depth. At this concentration, most of the spectral center-of-mass shift occurred within 1 mm. A further increase in the concentration would limit the penetration depth of SOCT applications, whereas a decrease in the concentration would reduce the amount of spectral centroid shift.

An agar sample was prepared with two distinct vertical columns separated by a glass wall to prevent diffusion between the columns. One column contained an absorbing agent concentration of 50  $\mu\text{g/mL}$ , and the other column contained no absorbing agent. An equal concentration of 0.2% Intralipid solution was added to both columns for use as a scattering agent. The resulting false-color hue-saturation SOCT image showed that the spectrum of the backreflected light from the column containing the absorbing agent had shifted toward longer wavelengths with increasing depth, whereas this effect was negligible for the column without the absorbing agent.

## SOCT Imaging of Tissue

A stalk of green celery (*Apium graveolens* var. *dulce*) was imaged by SOCT. A celery stalk was cut near the root, leaving the upper leaves intact to facilitate transpiration. The celery stalk contained two distinct tissue structures. The bulk of the stalk was composed of collenchyma tissue, in which most of the cells were relatively large in size with thickened cell walls that mechanically supported the stalk. Distributed around the center of the stalk were vascular bundles in which the cells were relatively smaller in size and formed conducting vascular tubes to transport water and nutrients between the roots and leaves. These tissues were observed using light microscopy, as shown in FIG. 9D, in which the vascular bundle is in the center and is surrounded by collenchyma tissue.

An absorbing agent mixture was prepared by combining 10 mL of a 50 µg/mL mixture of the NIR dye of Example 2 with 0.5 mL of a 200 µg/mL mixture of Rhodamine 5G. A control image of the celery stalk was taken with SOCT before application of the absorbing agent. This control image is shown in FIG. 9B. The root end of the stalk was then submerged in the absorbing agent mixture for 4 h to allow for capillary transportation. After the absorbing agent mixture had been transported, the stalk was imaged by SOCT at the same location as the control image. This contrast enhanced SOCT image is shown in FIG. 9A. The celery stalk was cut in cross section at the SOCT imaging location and imaged by fluorescence microscopy (FIG. 9C) and light microscopy (FIG. 9D). Fluorescence microscopy was permitted by the Rhodamine in the absorbing agent mixture. The single-photon absorption spectrum for Rhodamine was outside of the titanium:sapphire laser spectrum, and the two-photon absorption and emission efficiency was extremely low ( $<10^{-10}$ ), resulting in no detectable contribution to the SOCT signal.

When imaged by SOCT without an absorbing agent, no significant difference in the spectral center of mass was observed between the two types of tissues. The contrast between the two tissues was enhanced by the presence of the NIR absorbing agent. This contrast enhancement was apparent in the vascular regions containing the NIR absorbing agent, where strong shifting of the spectral centroid occurred. In FIG. 9A, the color bar represents the correspondence between pseudocolor labeling and the spectral centroid shift in the image. The surrounding avascular collenchyma tissue showed minimal changes. The vascular bundle region showing strong SOCT contrast enhancement also correlated well with the region showing strong fluorescence (FIG. 9C).

## Example 5

## Comparison of TFD Performance on Simulated SOCT Signals

Synthetic signals were generated in order to produce a comprehensive class of SOCT-like signals controlled by several parameters. Their design was based on the equation for the interferometric power spectrum  $I(\omega, z)$ :

$$I(\omega, z) = S(\omega) H_s(\omega, z) H_a(\omega, z) H_m(\omega)$$

To simplify the simulation parameters, the sampling time and reference arm translation speed were adjusted such that the 800 nm laser wavelength corresponded to a digital frequency of 0.125 Hz. Axial depth was converted to a signal acquisition time from 0 sec to 1 sec. Although an experimental OCT

system would acquire axial scans much faster, these numerically-simple parameters were used without losing theoretical generalities.

Three different imaging scenarios were considered. The first scenario was a Gaussian pulse with a spectrum centered at 800 nm and a FWHM of 100 nm. This synthetic signal corresponded to a typical SOCT signal from a perfectly-reflecting mirror, and was used for testing TFD performance on minimal time-frequency spread. The second scenario was two consecutive "spectrally absorbed" Gaussian pulses, in which the first pulse contained all of the frequencies of the optical source, and the second pulse contained only the lower half of the frequencies of the optical source. This scenario corresponded to two closely-spaced reflecting interfaces with different spectral reflection profiles. By varying the distance between the pulses, this scenario was used for testing the minimal spatial separation of TFDs given a prior requirement on frequency resolution. The third scenario was a consecutive Gaussian pulse sequence with random positioning and a slowly varying spectrum between pulses, representing a region of homogeneous absorption and scattering. The absorbing agents were assumed to uniformly absorb upper half frequencies, following Beer's law. This sequence corresponded to SOCT signals scattering back from tissue with a roughly uniform scattering agent distribution but with high absorbing agent concentrations, and was used for testing the ability of the TFDs to retrieve the absorption coefficient of the media.

The synthetic signals were subjected to TF analysis using different TFDs. The TFDs of the signal on the TF plane were generated as color-scale images. In the cases where the distribution has negative or complex values, the magnitude was taken. For each of the TFDs, parameters were optimized by extensive parameter searching to represent the best possible outcome using that type of TFD. In cases in which good criteria were difficult to obtain, such as when lowering the cross-terms compromised the resolution of the auto term, qualitative evaluation was used to produce the best analysis.

To compare the overall quality of the TFDs on the synthetic signal from the first scenario, two criteria were used. The first criterion was the time-frequency spread (by measuring standard deviation) of the TFDs. The second criterion computed the unitless TF "concentration" or "sharpness" using the equation:

$$C = \frac{\int \int_{-\infty}^{\infty} |TFD(t, f)|^4 dt df}{(\int \int_{-\infty}^{\infty} |TFD(t, f)|^2 dt df)^2}$$

which is the fourth power of the  $L_4$  norm divided by the squared  $L_2$  norm of the magnitude of the TFD. See D. L. Jones and T. W. Parks, *IEEE Transaction on Acoustics, Speech and Signal Processing*, 38, 2127-2135 (1990). The testing results of TFDs on this synthetic signal are listed in Table 1. The linear TFD examined was the STFT with a Hamming window ("STFT"); the Cohen's class TFDs examined were WVD and Morlet wavelet ("WT"); and a Gaussian model was used for the model-based TFD ("Model-based"). The WVD achieved the best time-frequency concentration. Because signal model was exactly known for synthetic signal, model based TFD completely recovered the ideal TFD.

TABLE 1

TF resolution of TFDs on synthetic signal from first imaging scenario					
	Ideal TFD	STFT	WT	WVD	Model-based
Time spread (s)	0.027	0.032	0.040	0.020	0.027
Frequency spread (Hz)	0.016	0.032	0.038	0.022	0.017
Time-frequency product ( $10^{-4}$ )	4.32	10.2	15.2	4.40	4.59
Concentration	250	102	132	305	250

To compare the overall quality of the TFDs on the synthetic signal from the second scenario, two neighboring scattering agents were considered to be distinct in SOCT if the maximum shift of the spectral centroid was at least half that of what the shift would be if the scattering agent was alone. The linear TFD examined was the STFT with a Hamming window ("STFT"); the Cohen's class TFDs examined were SPWVD and Morlet wavelet ("WT"); and Ideal LPFs and HPFs were used for the model-based TFDs ("Model-based"). Simple WVD did not perform well under this situation because of the strong cross-terms. Instead, the smoothed pseudo-Wigner-Villie distribution (SPWVD) was used with a smoothing Gaussian kernel applied independently in the time and frequency direction. The minimal distances needed for different TFDs to discriminate the two pulses are listed in Table 2. For reference, the structural OCT resolution (by FWHM criterion) is also listed in Table 2. The Cohen's-class TFDs had better performance than the STFT on this synthetic signal.

TABLE 2

TF resolution of TFDs on synthetic signal from second imaging scenario						
	Structural OCT	Ideal TFD	STFT	WT	SPWVD	Model-based
Minimal distance (s)	0.053	0.025	0.036	0.039	0.033	0.026

To compare the overall quality of the TFDs on the synthetic signal from the third scenario, the absorption was assumed to follow Beer's Law. The locations of the scattering agents were first identified by peak detection. Then, absorption spectra were determined from TFDs based on least-square curve fitting of TFDs from multiple scattering agents. The error function was calculated from the measured absorption spectra  $A'(f)$  and the expected absorption spectra  $A(f)$  using the formula:

$$\text{Error} = \sum_{\text{Frequency Band}} \frac{A'(f) - A(f)}{A(f)}$$

The "Frequency Band" was defined by the 10% level criterion. The linear TFD examined was the STFT with a Hamming window ("STFT"); the Cohen's class TFDs examined were SPWVD and Morlet wavelet ("WT"); and Ideal LPFs were used for the model-based TFDs ("Model-based"). The errors for different TFDs are listed in Table 3. The model-based TFD out-performed all other TFDs. Linear TFDs were reasonably good, while all Cohen's-class TFDs gave erroneous outcomes due to cross-terms and non-ideal smoothing operations.

TABLE 3

TF resolution of TFDs on synthetic signal from third imaging scenario					
	Ideal TFD	STFT	WT	SPWVD	Model-based
Error	0.0%	-5.0%	-6.1%	34.3%	0.0%

## Example 6

## Comparison of TFD Performance on Experimental SOCT Signal of Two Closely-Spaced Reflecting Interfaces

Experimental SOCT signals were obtained that corresponded to the second imaging scenario in Example 5. A sample was constructed to provide two back-scattering interfaces that were spatially close and that exhibited different back-scattering spectra. Double-sided tape having a thickness of approximately 80  $\mu\text{m}$  was placed between and along one edge of two 24 $\times$ 60 mm glass coverslips. A paper clip compressed the coverslips at the opposite edge to make a semi-closed thin gap between the two coverslips. The assembly was then turned vertically and one wedge-shaped open side was submerged into a shallow 20 mg/ml solution of the near-infrared dye of Example 2. After a few seconds, the absorbing agent solution filled the wedge-shaped space between the coverslips via capillary forces. Unlike many other water-soluble NIR absorbing agents, this dye strictly followed the Beer's Law of absorption up to very high concentrations. Even at 20 mg/ml, the absorbing agent still maintained its expected absorption spectrum. No photobleaching effect was observed with 10 mW of focused laser power over a period of 10 minutes.

The sample was imaged with a fiber-based OCT setup similar to the device of Example 1, but with the following modifications. A thin lens with a 40 mm focal length was used to minimize the effect of chromatic aberration, dispersion, and focusing. Non-linearities in the reference scanning rate were accounted for by acquiring a reference fringe pattern using a narrowband laser diode with a center wavelength around 776 nm and a bandwidth of 1 nm, and applying a data correction algorithm. This OCT system provided 4  $\mu\text{m}$  axial resolution, with a 3.2 mm depth of focus (confocal parameter) in air. The interference was detected using an auto-balancing detector (Model 2007, New Focus, Inc.). The signal was amplified and filtered using an anti-aliasing low-pass filtered in a custom analog circuit. Before applying TFD analysis, the signal was bandpass filtered to remove excessive noise in the digital domain and was digitally corrected for dispersion.

Axial scans along different wedge positions (different absorbing agent thicknesses) were acquired. The sample was placed on an angle-adjustable stage such that the light reflected back from the glass/liquid interfaces was in a near-normal direction. The incident laser power was attenuated to prevent saturation at the photodetector. The interference fringe data were collected for analysis with different TFDs. The interference fringes resulting from multiple reflections (light bouncing back and forth between the two glass interfaces) were found have magnitudes at least 50 times smaller than the main interference fringes, and therefore were not used in our analysis.

The windows chosen for the STFT were Hamming windows of length corresponding to one coherence length of the incident laser. The actual distance between the two interfaces



in terms of coherence lengths was measured by counting the number of fringe peaks between two pulse centers and the number of fringe peaks between the FWHM from a single pulse off of a mirror. Most of the shorter wavelengths were absent from the light reflected from the lower absorbing agent/glass interface because of the absorbing agent absorption. Blurring of the time-frequency representation as the separation of the two interfaces narrowed was observed, as would be expected from the "uncertainty principle". Specifically, when the distance between the two interfaces was less than the coherence length of the optical source, it became difficult to resolve them.

The experimental signals were subjected to TF analysis using different TFDs to compare the resolving power of the TFDs in this setting. The STFT, Scalogram, Choi-William distribution, and model-based TFDs were examined. The length of time windowing for the STFT and the Choi-William distribution was chosen to correspond to 1  $\mu\text{m}$  in air. This length offered the best separation by qualitative assessment. Morlet wavelets were chosen for the Scalogram. The model for the model-based TFD was set up by assuming that the TFD of the pulse from the first interface was the same as the WVD of a pulse from a mirror ( $\text{TFD}_M(z, I)$ ) except for a scaling factor, and that the TFD of the pulse from the second interface was the first TFD after absorbing agent absorption multiplied by another scaling factor:

$$\text{TFD} = A \times \text{TFD}_M(z, \lambda) + \text{TFD}_M(z - z_i, \lambda) \exp(-B \times e(I))$$

where  $A$  and  $B$  were the scaling factors and  $z_i$  was the distance between two interfaces. This equation was digitized in  $z$  and  $\lambda$  to have each  $z$  point represent 0.1  $\mu\text{m}$  and each  $\lambda$  point represent 1 nm. The term  $e(I)$  representing absorbing agent absorptivity was measured by a spectrometer. Spline interpolations were used whenever the experimentally measured data had different data points from the model. The criterion for model optimization was to search for the best  $A$ ,  $B$ , and  $z_i$  such that the lowest mean-square-error between the model TFD and the TFD by STFT was generated. Because it was computationally-expensive to search for three optimal parameters ( $A$ ,  $B$ ,  $z_i$ ) in 3-D space,  $z_i$  was first determined based on the fringe number. The parameters  $A$  and  $B$  were only searched for their optimal values in 2-D space, and then the optimal  $z_i$  was determined for that  $A$  and  $B$ . The two-step recursion was repeated until results stabilized.

The TFDs from Cohen's class (the Choi-William distribution and Scalogram) had comparative performance, while both performed better than the STFT. Artifacts in the TFD plots for the Choi-William distribution were due to the cross-terms during the bilinear transformation of the signal. However, because the cross-terms were out of the primary signal bands, they could be rejected easily. Confirming the results of the simulation of Example 5, the model-based TFD had the best performance in terms of sharpness, although it may or may not have represented the true TFD.

#### Example 7

##### Comparison of TFD Performance on Experimental SOCT Signal of Homogeneous Media Containing Small Number of Scattering Agents

Experimental SOCT signals were obtained that corresponded to the third imaging scenario in Example 5. Phantom samples were prepared in liquid form to provide homogeneous media containing a small amount of scattering agents. The near-infrared dye ADS830WS (American Dye Sources,

Inc.) was used. Unlike the absorbing agent SDA7460 used in Example 6, this dye had a sharp absorption peak around 810 nm, which was close to the emission spectrum of the laser source. Having an absorption peak near the center of the laser source spectrum facilitated the evaluation of the performance of different TFDs. When dissolved in methanol, this absorbing agent was also very stable and did not show any photobleaching effect under 10 mW of focused laser power over a period of 10 minutes. Silica microbeads 0.33  $\mu\text{m}$  in diameter (Bang Laboratories, Inc.) were used as scattering agents. The solution containing the absorbing agent and microbeads was placed inside a thin glass cuvette and imaged with the SOCT setup of Example 6. The concentrations of the absorbing agent and silica microbeads were adjusted such that the absorption loss was 5 times larger than the scattering loss at 800 nm.

Prior to SOCT imaging, the mixture was measured by a spectrometer for the combined effect of absorption loss and scattering loss. The absorption spectra were retrieved by each TFD method similar to the analysis on the third synthetic signal in Example 5, except for three additional modifications. First, a control sample containing the same concentration of microbeads, but without absorbing agent, was used for data-correction to reduce the system error. Second, because very closely-spaced scattering agents exhibit a significant spectral-interference effect, averaging of TFDs from 512 scan lines was performed to obtain the final TFDs. Third, because of the large number of data points collected (50,000 points/scan line), it was not possible to perform different TFDs directly without significant computational complexity. Therefore, taking advantage of the fact that the SOCT signals were narrow pass-band signals, data were demodulated and decimated to obtain the shortest possible analytic signals without losing frequency information within the laser source spectrum. The time window sizes for the STFT, Choi-William distributions and the model based TFDs were chosen to be four coherence length. The Morlet wavelet was used for the wavelet transform. Because no prior information was assumed, an autoregressive (AR) model using the Burg method was used for the model-based TFDs, with a model order set to 4.

The absorption spectra obtained by different TFDs are shown in FIG. 10, together with the spectral range of the laser (FWHM). For comparison, each spectra was normalized to its respective peak value. In this SOCT imaging scenario, the STFT and the wavelet transform were the reliable methods. The model-based TFD had reasonably good performance even though no assumption was made when constructing the model. The spectrum retrieved using the Choi-William TFD was totally random. These results confirm the predictions of the simulation of Example 5.

#### Example 8

##### Spectroscopic Spectral-Domain OCT Imaging

A custom-designed and constructed multi-modality microscope was used in this study, which enabled not only OCT and SOCT, but also simultaneous multi-photon microscopy using the same optical source. The light source consisted of a frequency-doubled Nd:YVO<sub>4</sub>-pumped Ti:sapphire laser with a center wavelength of 800 nm, a bandwidth of 40 nm, and an 80 MHz pulse repetition rate. This source was used as both a low-coherence source for OCT and also as an excitation source for multi-photon microscopy. The microscope objective (20 $\times$ , 0.95 NA, water immersion, Olympus) had a high NA in order to achieve high lateral resolution and tight spatial

confinement of the backscattered OCT signal. Dispersion in the proprietary glass of the objective was balanced digitally in the acquired image data. The interferometric setup was similar to those used in spectral-domain OCT. In our configuration, a free-space 50/50 beam splitter was used. The light in the detection arm was collimated and dispersed off a blazed diffraction grating having 830.3 grooves per millimeter. The optical spectrum was focused on a line-scan camera (L104k-2k, Basler, Inc.) which contained a 2048-element CCD array of detection elements with a maximum readout rate of 29 kHz. Digital processing of the detected signal included a Spline interpolation to make the signal more uniform, and a discrete Fourier transform on each set of 2048, 10-bit, values captured by the CCD to transform the signal from the frequency (spectral) domain into the spatial (depth) domain.

The axial PSF of the objective using spectral-domain OCT detection (coherence-gating) was measured to be 2.2  $\mu\text{m}$  at FWHM. Because the source spectrum was roughly Gaussian, the sensitivity of OCT to the retro-reflected light decreased exponentially with axial distance. Note that in this system the confocal gating (confocal parameter=2.2  $\mu\text{m}$ ) was below the coherence gating (coherence length=7  $\mu\text{m}$ ) with the laser source bandwidth of 40 nm. OCT images of a calibrated U.S. Air Force test target were used to determine the high transverse resolution of this system. By use of the edge-scan definition, a transverse resolution of less than 0.9  $\mu\text{m}$  was measured. To determine the sensitivity of the system, the OCT PSF from a mirror translated through the focal plane was measured with calibrated attenuation filters inserted in the sample arm. The SNR was calculated by taking the ratio between the signal power and the noise variance. With 1 mW (0 dBm) of power incident on the mirror, the measured SNR was found to be equal to 97 dB. The dynamic range within experimental image data was approximately 60 dB. Calibrated fluorescent microbeads were used to determine the axial and transverse multi-photon microscopy resolutions of our system, which were 0.8  $\mu\text{m}$  and 0.5  $\mu\text{m}$ , respectively. Incident optical power ranged from 1-5 mW (1 mW typical), with the higher power used to excite two-photon fluorescence from green-fluorescent protein (GFP).

The spectral-domain OCT interference fringes were acquired at 2048 pixels per OCT point, covering a potential full-array light spectral range from 740 nm to 860 nm, and which corresponded to an imaging depth of approximately 2.7 mm in air. The raw spectral-domain OCT interference was given by:

$$I(k, z)_{z=z_0} = 2[R_s R_r(k, z)_{z=z_0}]^{1/2} S(k) \cos(2k\Delta p)$$

where  $k$  is the free-space wave number,  $z$  is the depth,  $R_r$  and  $R_s$  are the reference reflectivity and sample reflectivity, respectively,  $S(k)$  is the source spectral density, and  $\Delta p$  is the optical pathlength difference at  $z_0$  that is defined by the focal gating of the high NA objective. The reference reflectivity  $R_r$  was assumed to be wavelength-independent. The modulation transfer function of the spectral-domain OCT system was calibrated using a mirror, and the raw spectral domain signal was re-mapped to  $k$  space using cubic Spline interpolation. The spectral-domain data then was demodulated to baseband by first taking the fast Fourier transform (FFT) to obtain the depth-dependent analytical signal, followed by the inverse FFT of the depth signal segment centered around the focal gate position. A Gaussian window of 512 points with a FWHM of 256 points was used, which corresponded to a spectral resolution of 0.5 nm.

The retrieved  $R_s(k)$  at the focal plane of the OCT objective was processed by two different SOCT analysis methods. The

first method was based on metameric imaging, where the scattering spectrum is divided into different sub-spectral bands. The signal intensity in each sub-spectral band was integrated to produce the intensity for one color channel. For this study, the window within the FWHM of the source spectrum was divided into three equally spaced sub-bands, and the intensity from the low-, mid-, and high-frequency bands were assigned to the red, green, and blue channels, respectively. This method represented similar information as the traditional spectral centroid method, but was more robust and more similar to the mechanism of human vision. The second method was based on spectral analysis initially proposed in LSS. The back-scattered spectra were first analyzed by the FFT, and the first peak of the FFT data was used for hue information in an HSV color scale. This peak position was related to the physical size and inter-scatterer distance of the dominant scatterers, such as the nucleus, within the focal gate at that location. The metameric method was more qualitative and suited for attenuation-based measurements in SOCT, while the spectral analysis method used in LSS was more quantitative and suited for scattering-based measurements in SOCT. These representative SOCT analysis methods were performed on spectral-domain OCT data collected from tissue and cell specimens imaged using our multi-modality microscope.

FIG. 11 shows image-data acquired from mammary tissue of a rat, consisting of adjacent adipose (fat) and muscle tissue. FIG. 11B is a histological image of the tissue. The high-resolution OCT image showed individual adipocytes in the center of the image (FIG. 11A), but exhibited regions of low backscatter over the more dense muscle tissue at the upper right and lower left corners of the image, possibly due to forward scattering or polarization-dependent effects. However, compared to OCT, the SOCT analysis methods (FIGS. 11C and 11D, respectively) showed increased contrast for muscle compared to the adipose tissue where there was sufficient backscattered signal for spectral analysis. This contrast enhancement (light yellow and blue regions in FIG. 11C, and green and blue regions in FIG. 11D) was due to different scatterer sizes and scatterer organization (likely nuclei and other organelles), and was more prominent in the SOCT image based on LSS spectral analysis (FIG. 11D, green regions), which has been shown to detect changes in nuclear regions.

FIG. 12 shows an OCT image of live fibroblast cells in culture, and the corresponding SOCT image using LSS spectral analysis (FIGS. 12A and 12B, respectively). The first peak-positions of the FFT obtained from the modulation patterns of the back-scattered light were clearly different near the center of the cell, compared to the periphery of the cell. One possible reason for this difference was the presence a large scatterer, the nucleus, located near the center of these cultured cells. These SOCT findings were confirmed by multi-photon imaging of this cell culture, using the simultaneous multi-modality capabilities of the microscope. These transfected fibroblasts expressed GFP-labeled vinculin (a cell adhesion protein) and were co-labeled with a DNA-specific dye (Hoechst 33342) for localization of nuclei relative to the surrounding cell structures (FIG. 12C). The simultaneous multi-modality imaging afforded by the microscope enabled overlays of various image channels, as shown for OCT and the multi-photon fluorescence from the DNA/nuclear dye (FIG. 12D). The SOCT analysis information was consistent with the multi-photon imaging data in identifying the locations of the nuclei within these cells. Of the six nuclei identified in the multi-photon fluorescence image (FIG. 12C), five nuclei were clearly identified in the SOCT image (FIG. 12B,

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green/blue regions). The remaining cell nucleus (left-most cell) may not be identified as clearly with SOCT because this cell may have been smaller and had a flatter profile than the others, resulting in a backscattering spectrum more similar to the background.

In conclusion, spectroscopic spectral-domain OCT analysis with tight focal gating decoupled the inherent trade-off between spectral and spatial (depth) resolution. This enabled the extraction of more minute spectroscopic features from within the small imaging volumes, making localized analysis of wavelength-dependent scattering possible. Wavelength-dependent scattering and the resulting spectral modulation were information-rich processes that were dependent on both optical properties of the scatterer and the inter-scatterer spacing. Spectroscopic spectral-domain OCT was capable of enhancing contrast in various tissues and cells based solely on endogenous structures.

While various embodiments of the invention have been described, it will be apparent to those of ordinary skill in the art that other embodiments and implementations are possible within the scope of the invention. Accordingly, the invention is not to be restricted except in light of the attached claims and their equivalents.

What is claimed is:

1. A method of forming an image of tissue, comprising: selecting at least one contrast agent, comprising at least one water-soluble, biocompatible absorbing agent having an absorption profile; delivering the contrast agent to the tissue; acquiring spectroscopic optical coherence tomography data from the tissue across a spectrum range, wherein the acquiring does not change the absorption profile of the absorbing agent; and converting the spectroscopic optical coherence tomography data into at least one image, wherein the at least one absorbing agent absorbs in the near-infrared region, the absorption profile of the absorbing agent is sharp, and the absorbing agent is highly absorbing in a first portion of the spectrum range and has little to no absorption in a second portion of the spectrum range.
2. The method of claim 1, wherein the at least one contrast agent further comprises at least one scattering agent.
3. The method of claim 1, wherein the selecting at least one contrast agent comprises: determining the optical window of the tissue; selecting a laser spectrum range that is within the optical window; and selecting at least one absorbing agent that absorbs within the laser spectrum range.
4. The method of claim 3, wherein the selecting at least one absorbing agent comprises selecting at least one absorbing agent having an absorbance spectrum that overlaps the laser spectrum range.
5. The method of claim 3, wherein the selecting at least one absorbing agent further comprises selecting at least one absorbing agent that can be delivered to the tissue.
6. The method of claim 3, further comprising selecting at least one scattering agent that scatters within the laser spectrum range.
7. The method of claim 6, wherein the selecting at least one scattering agent further comprises selecting at least one scattering agent that can be delivered to the tissue.
8. The method of claim 1, wherein the converting of the spectroscopic optical coherence tomography data into at least one image comprises:

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- performing time-frequency analysis on the data;
- performing spectral/pattern analysis on the data;
- retrieving the spatial distribution of the at least one contrast agent in the tissue; and
- correlating the spatial distribution with at least one display parameter.
9. The method of claim 8, wherein the at least one contrast agent further comprises at least one scattering agent, the retrieving comprises retrieving the spatial distributions of the at least one absorbing agent and of the at least one scattering agent, and the correlating comprises correlating the spatial distributions of the least one absorbing agent and of the at least one scattering agent with at least one display parameter.
10. The method of claim 1, wherein the tissue is living.
11. A method of forming an image of tissue, comprising: selecting a laser spectrum range that is within an optical window; selecting at least one water-soluble, biocompatible absorbing agent having an absorption profile, the absorbing agent absorbing within the laser spectrum range; selecting at least one scattering agent that scatters within the laser spectrum range; delivering the at least one absorbing agent and the at least one scattering agent to the living tissue; acquiring spectroscopic optical coherence tomography data from the living tissue across the spectrum range, wherein the acquiring does not change the absorption profile of the absorbing agent; performing time-frequency analysis on the data; performing spectral/pattern analysis on the data; retrieving the spatial distributions of the at least one absorbing agent and of the at least one scattering agent in the living tissue; and correlating the spatial distributions with at least one display parameter, wherein the at least one absorbing agent absorbs in the near-infrared region, the absorption profile of the absorbing agent is sharp, and the absorbing agent is highly absorbing in a first portion of the spectrum range and has little to no absorption in a second portion of the spectrum range.
12. A method of converting spectroscopic optical coherence tomography data into at least one image, comprising: performing time-frequency analysis on spectroscopic optical coherence tomography data from tissue; performing spectral/pattern analysis on the spectroscopic optical coherence tomography data; retrieving the spatial distribution of at least one contrast agent in the tissue; and correlating the spatial distribution with at least one display parameter, wherein the at least one contrast agent comprises at least one absorbing agent having an absorption profile, the acquiring does not change the absorption profile of the absorbing agent, spectroscopic optical coherence tomography data is acquired across a spectrum range, the at least one absorbing agent absorbs in the near-infrared region, the absorption profile of the absorbing agent is sharp, and the absorbing agent is highly absorbing in a first portion of the spectrum range and has little to no absorption in a second portion of the spectrum range.
13. The method of claim 12, wherein the at least one contrast agent comprises at least one scattering agent.
14. The method of claim 12, wherein the at least one contrast agent further comprises at least one scattering agent,

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the retrieving comprises retrieving the spatial distributions of the at least one absorbing agent and of the at least one scattering agent, and  
 the correlating comprises correlating the spatial distributions of the least one absorbing agent and of the at least one scattering agent with at least one display parameter. 5

**15.** A method of analyzing tissue, comprising:  
 delivering at least one contrast agent comprising at least one absorbing agent having an absorption profile, to the tissue; and 10  
 acquiring spectroscopic optical coherence tomography data from the tissue across a spectrum range,  
 wherein the acquiring does not change the absorption profile of the absorbing agent,  
 the absorption profile of the absorbing agent is sharp, and 15  
 the absorbing agent is highly absorbing in a first portion of the spectrum range and has little to no absorption in a second portion of the spectrum range.

**16.** A method of forming an image of tissue, comprising:  
 delivering at least one contrast agent comprising at least one absorbing agent having an absorption profile, to the tissue; 20  
 acquiring spectroscopic optical coherence tomography data from the tissue across a spectrum range,  
 wherein the acquiring does not change the absorption profile of the absorbing agent; 25  
 separating the signal due to absorption from the signal due to scattering; and  
 converting the spectroscopic optical coherence tomography data into at least one image, 30  
 wherein the absorption profile of the absorbing agent is sharp, and  
 the absorbing agent is highly absorbing in a first portion of the spectrum range and has little to no absorption in a second portion of the spectrum range. 35

**17.** The method of claim **16**, wherein the at least one contrast agent further comprises at least one scattering agent.

**18.** The method of claim **16**, wherein the converting the spectroscopic optical coherence tomography data into at least one image comprises: 40  
 performing time-frequency analysis on the data;  
 performing spectral/pattern analysis on the data;  
 retrieving the spatial distribution of the at least one contrast agent in the tissue; and

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correlating the spatial distribution with at least one display parameter.

**19.** The method of claim **18**, wherein the at least one contrast agent further comprises at least one scattering agent, the retrieving comprises retrieving the spatial distributions of the at least one absorbing agent and of the at least one scattering agent, and  
 the correlating comprises correlating the spatial distributions of the least one absorbing agent and of the at least one scattering agent with at least one display parameter.

**20.** The method of claim **16**, wherein the tissue is living.

**21.** A method of converting spectroscopic optical coherence tomography data into at least one image, comprising:  
 performing time-frequency analysis on spectroscopic optical coherence tomography data from tissue;  
 performing spectral/pattern analysis on the spectroscopic optical coherence tomography data;  
 retrieving the spatial distribution of at least one contrast agent comprising at least one absorbing agent having an absorption profile, in the tissue; and  
 correlating the spatial distribution with at least one display parameter;  
 wherein the performing spectral/pattern analysis on the spectroscopic optical coherence tomography data comprises separating the signal due to absorption from the signal due to scattering,  
 the spectroscopic optical coherence tomography data is acquired across a spectrum range without changing the absorption profile of the absorbing agent,  
 the absorption profile of the absorbing agent is sharp, and  
 the absorbing agent is highly absorbing in a first portion of the spectrum range and has little to no absorption in a second portion of the spectrum range.

**22.** The method of claim **21**, wherein the at least one contrast agent comprises at least one scattering agent.

**23.** The method of claim **21**, wherein the at least one contrast agent further comprises at least one scattering agent, the retrieving comprises retrieving the spatial distributions of the at least one absorbing agent and of the at least one scattering agent, and  
 the correlating comprises correlating the spatial distributions of the least one absorbing agent and of the at least one scattering agent with at least one display parameter.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 7,725,169 B2  
APPLICATION NO. : 11/405005  
DATED : May 25, 2010  
INVENTOR(S) : Stephen A. Boppart

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Page 3

First column, line 26, please delete "8994" and insert --89-94--.

First column, line 64, please delete "confiuration" and insert --configuration--.

Page 4

Second column, line 27, please put in vivo in italics.

Second column, line 43, please put in Vivo in italics.

Page 6

Second column, line 31, please put in vivo in italics.

Page 7

Column 1, line 11, please put in vivo in italics.

Column 1, line 39, please put in vitro in italics.

Column 2, line 11, please put in vivo in italics.

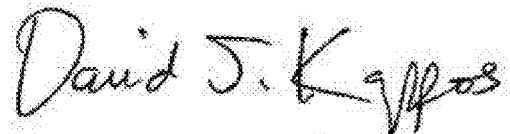
Page 8

Column 2, line 22, please delete "imaing" and insert --imaging--.

Page 9

Column 1, line 10, please delete "B<sup>1</sup> and D<sup>1</sup>" and insert -- $B^1 \prod_i$  and  $D^1 \sum_i^+$ --.

Signed and Sealed this  
Tenth Day of April, 2012



David J. Kappos  
Director of the United States Patent and Trademark Office