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(54) **CLASSIFYING IMAGE FEATURES**

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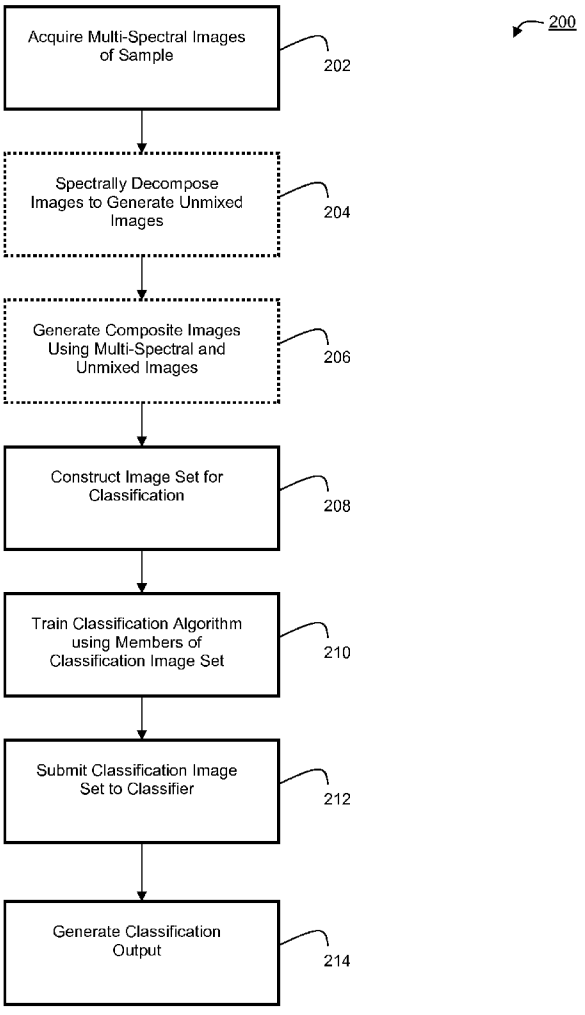
USPC **382/228**

(57) **ABSTRACT**

Methods are disclosed for classifying different parts of a sample into respective classes based on an image stack that includes one or more images.



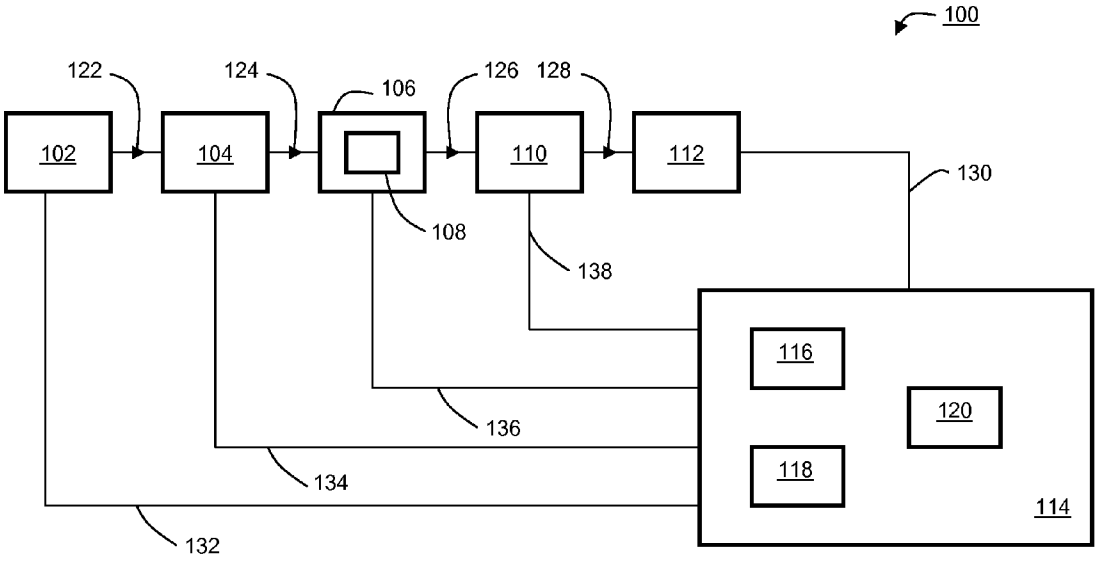

100
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FIGURE 1

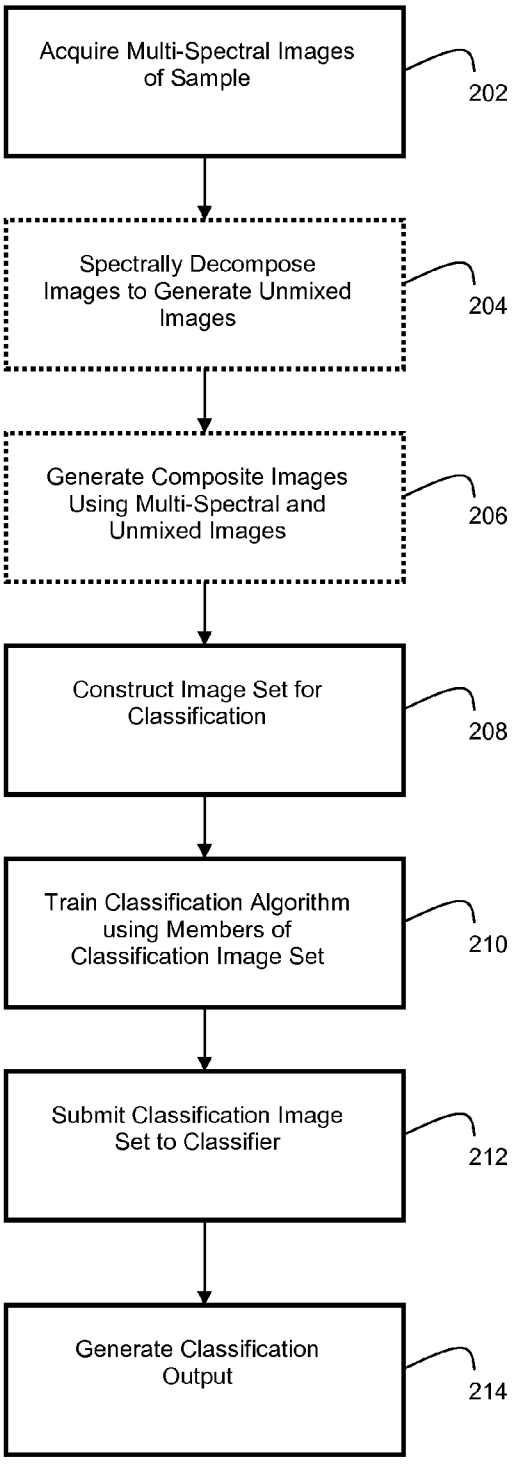
200
Acquire Multi-Spectral Images of Sample
202
Spectrally Decompose Images to Generate Unmixed Images
204
Generate Composite Images Using Multi-Spectral and Unmixed Images
206
Construct Image Set for Classification
208
Train Classification Algorithm using Members of Classification Image Set
210
Submit Classification Image Set to Classifier
212
Generate Classification Output
214

FIGURE 2

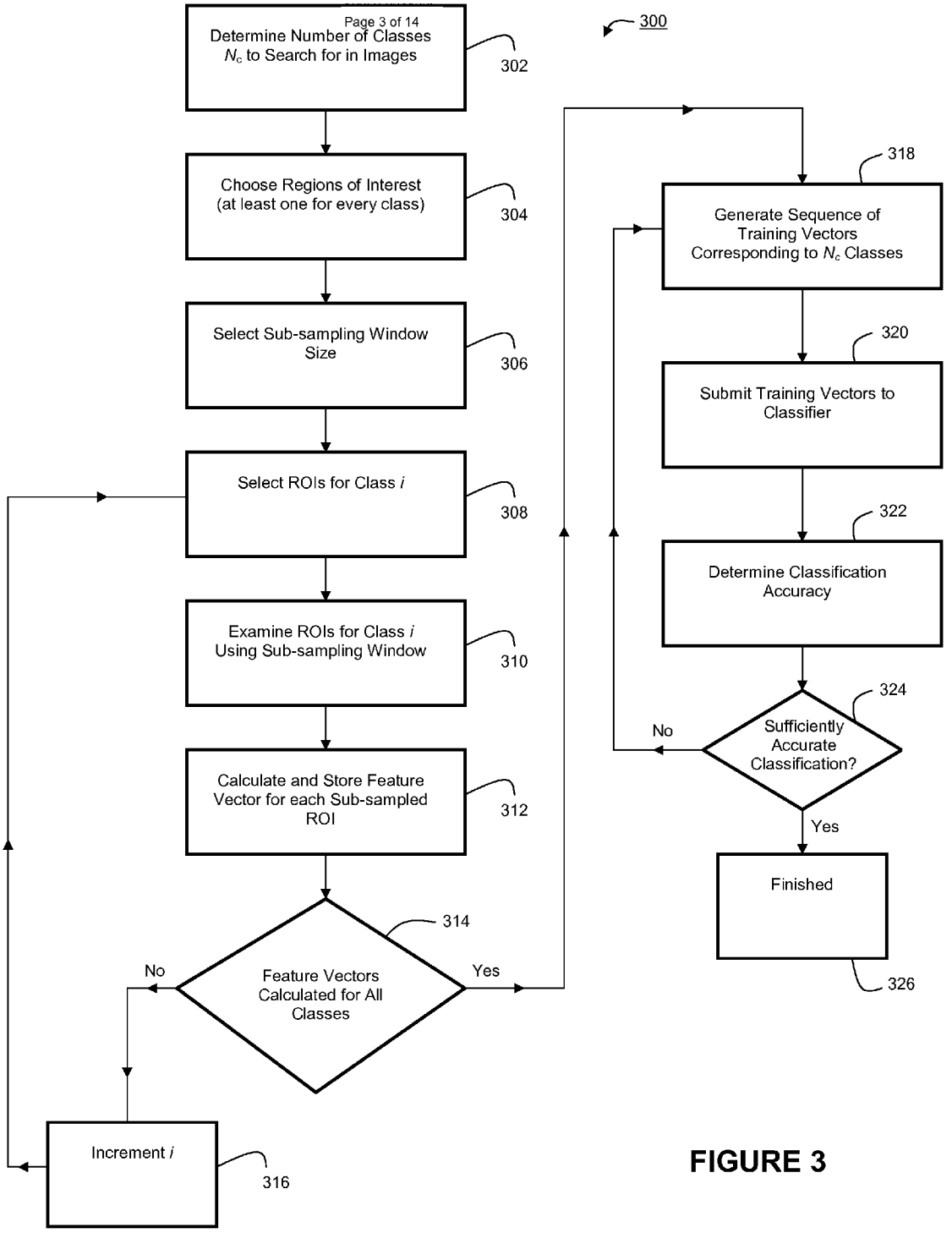
300
Page 3 of 14
Determine Number of Classes N_c to Search for in Images
302
Choose Regions of Interest (at least one for every class)
304
Select Sub-sampling Window Size
306
Select ROIs for Class *i*
308
Examine ROIs for Class *i* Using Sub-sampling Window
310
Calculate and Store Feature Vector for each Sub-sampled ROI
312
Feature Vectors Calculated for All Classes
314
No
Yes
Increment *i*
316
Generate Sequence of Training Vectors Corresponding to N_c Classes
318
Submit Training Vectors to Classifier
320
Determine Classification Accuracy
322
Sufficiently Accurate Classification?
324
No
Yes
Finished
326

FIGURE 3

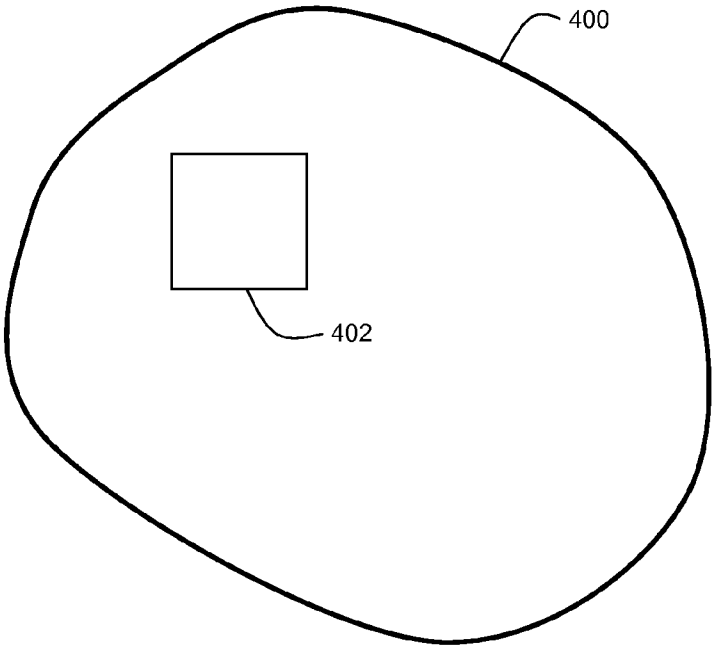

FIGURE 4

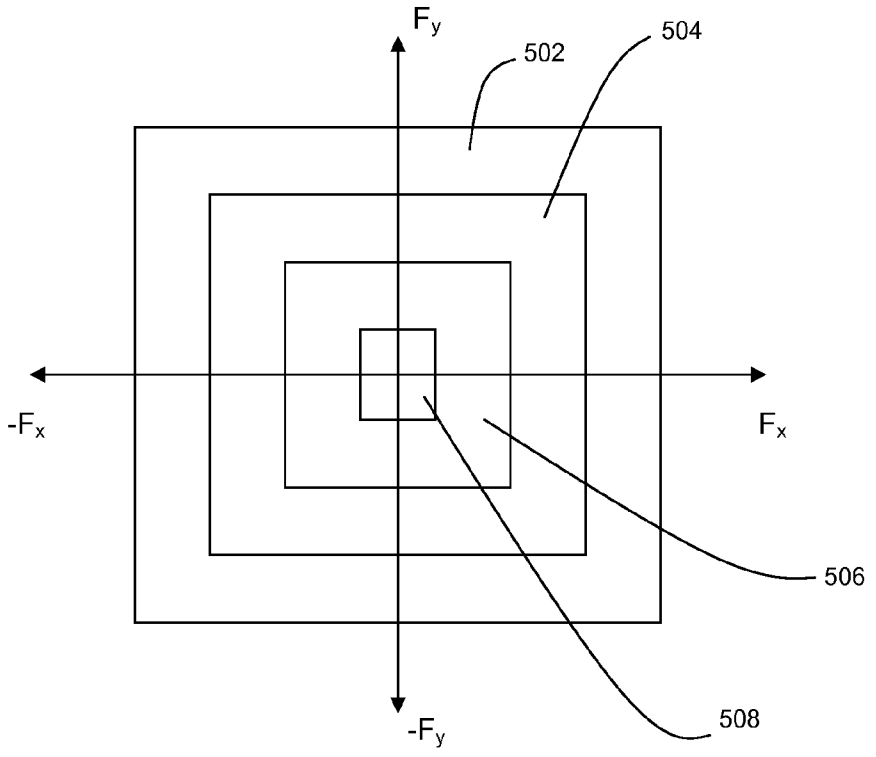

FIGURE 5

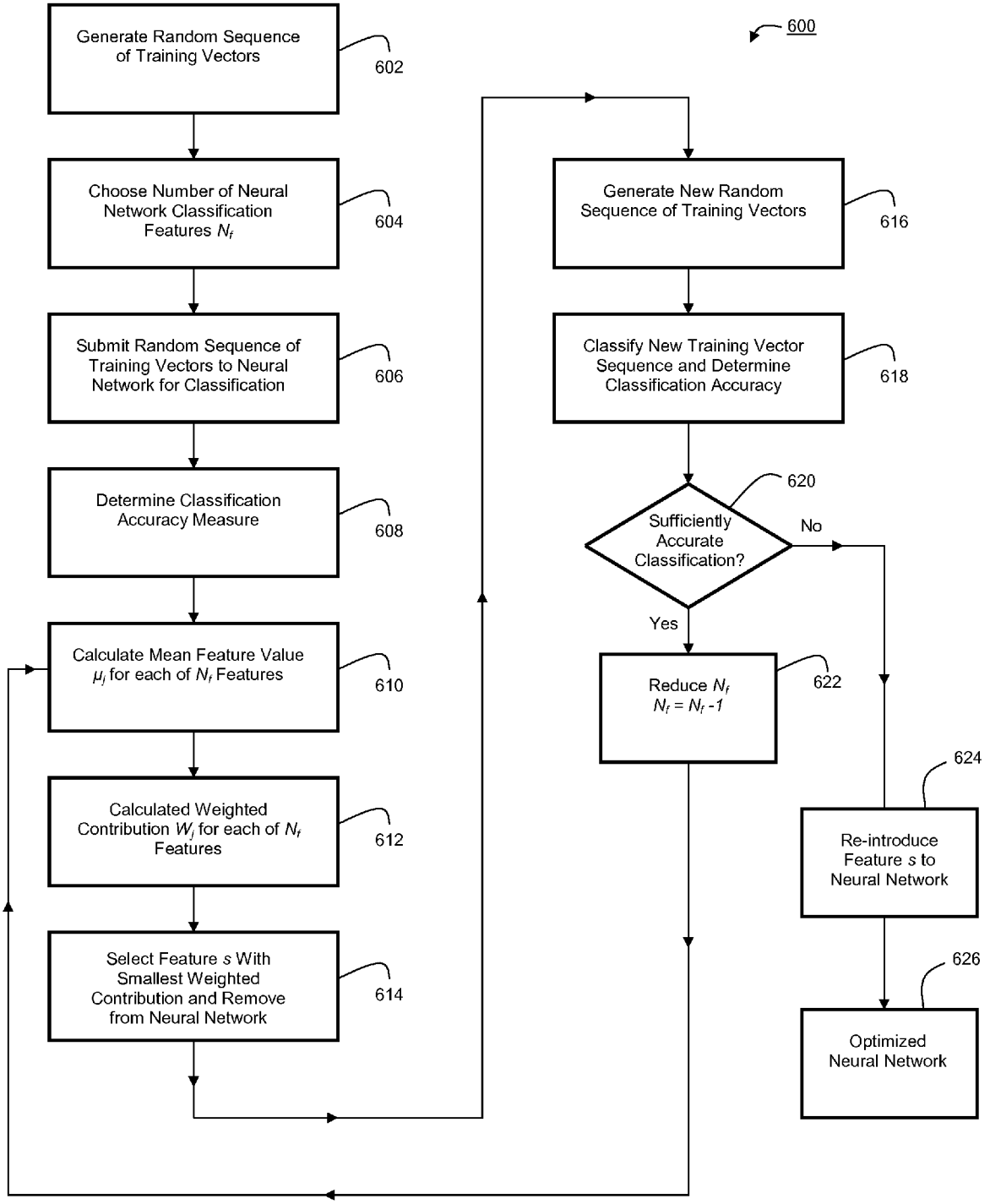
600
Generate Random Sequence of Training Vectors
602
Choose Number of Neural Network Classification Features N_f
604
Submit Random Sequence of Training Vectors to Neural Network for Classification
606
Determine Classification Accuracy Measure
608
Calculate Mean Feature Value μ_j for each of N_f Features
610
Calculated Weighted Contribution W_j for each of N_f Features
612
Select Feature s With Smallest Weighted Contribution and Remove from Neural Network
614
Generate New Random Sequence of Training Vectors
616
Classify New Training Vector Sequence and Determine Classification Accuracy
618
620
Sufficiently Accurate Classification?
No
Yes
Reduce N_f $N_f = N_f - 1$
622
624
Re-introduce Feature s to Neural Network
626
Optimized Neural Network

FIGURE 6

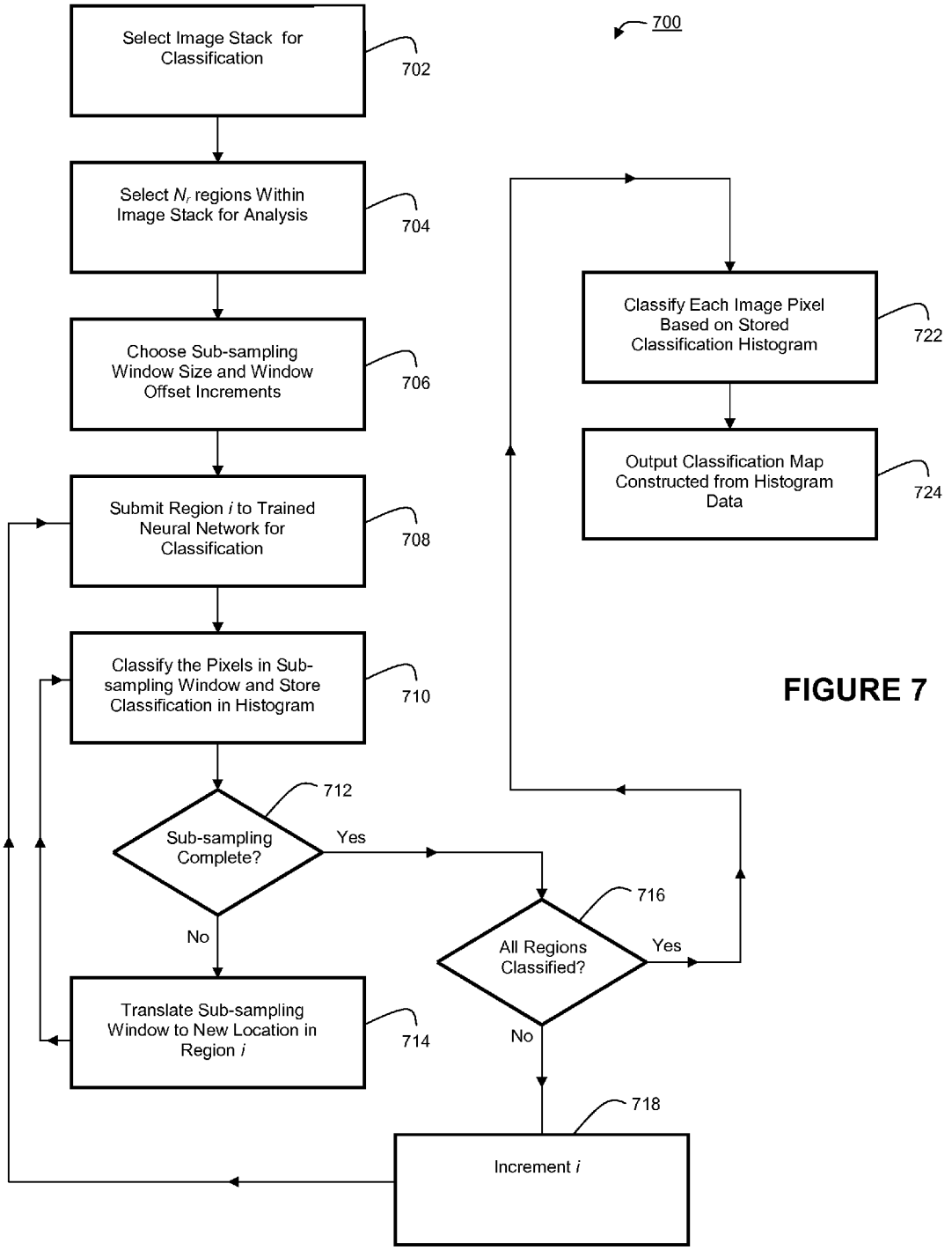
700
Select Image Stack for Classification
702
Select N_r regions Within Image Stack for Analysis
704
Choose Sub-sampling Window Size and Window Offset Increments
706
Submit Region *i* to Trained Neural Network for Classification
708
Classify the Pixels in Sub-sampling Window and Store Classification in Histogram
710
712
Sub-sampling Complete?
Yes
No
Translate Sub-sampling Window to New Location in Region *i*
714
716
All Regions Classified?
Yes
No
718
Increment *i*
Classify Each Image Pixel Based on Stored Classification Histogram
722
Output Classification Map Constructed from Histogram Data
724

FIGURE 7

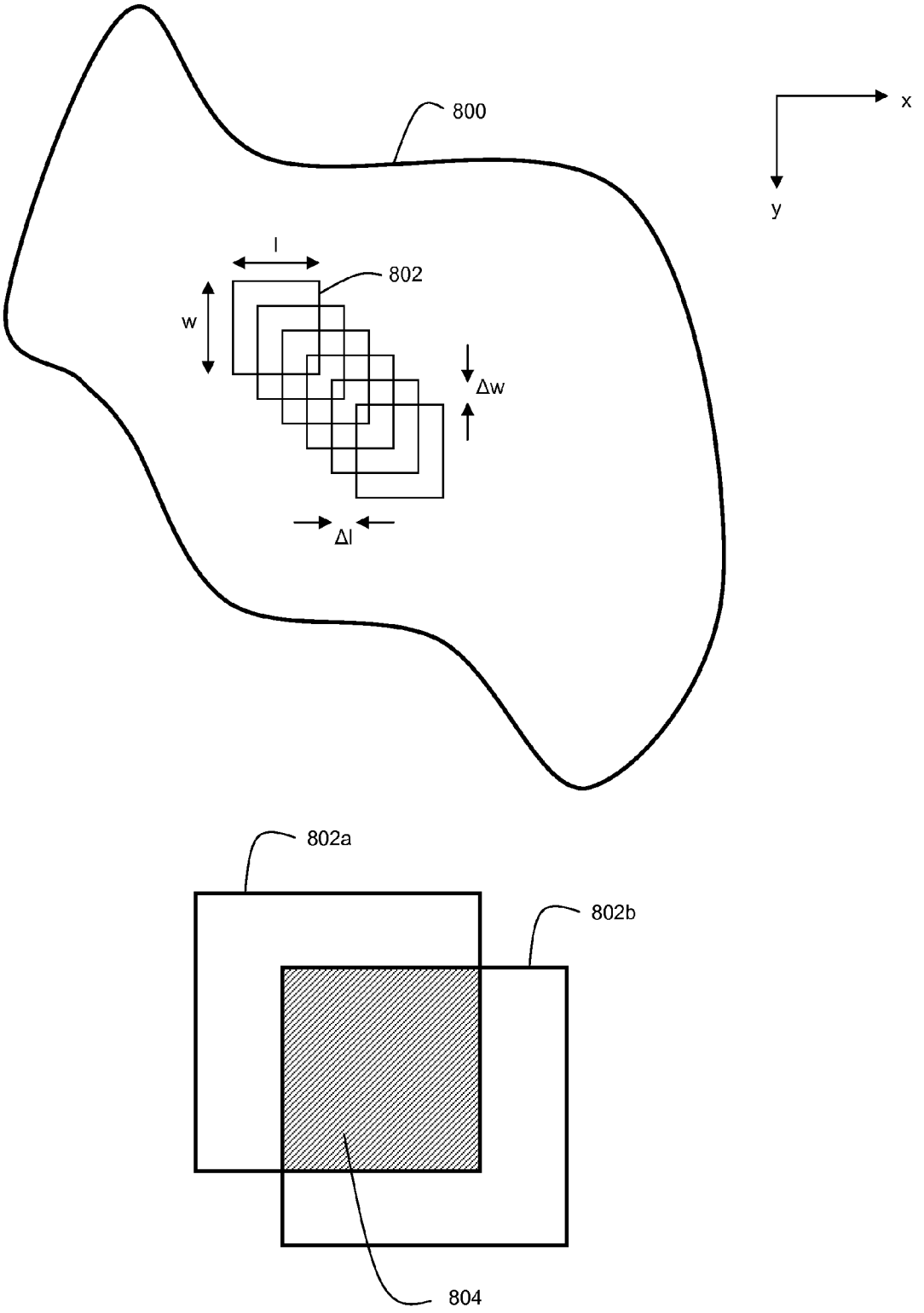

FIGURE 8

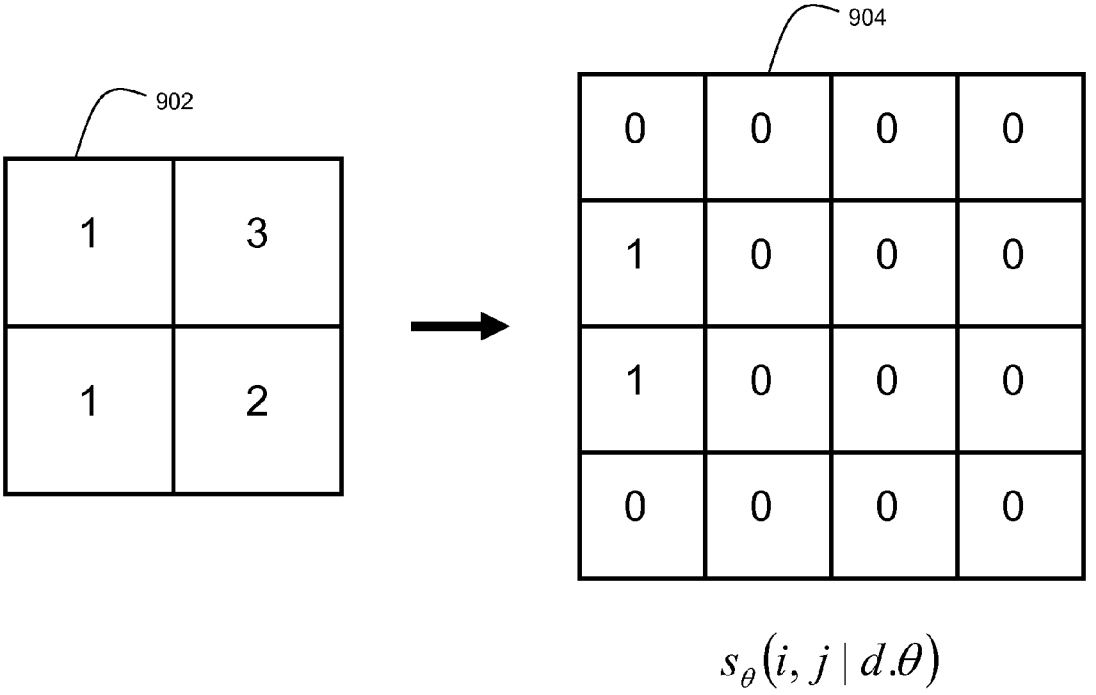

FIGURE 9

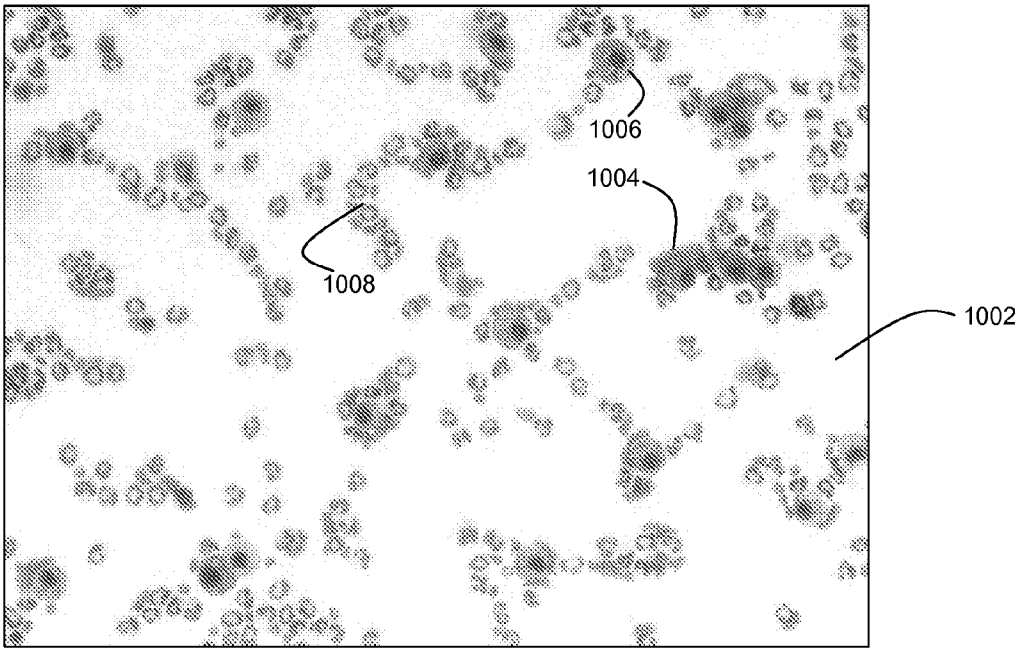

FIGURE 10A

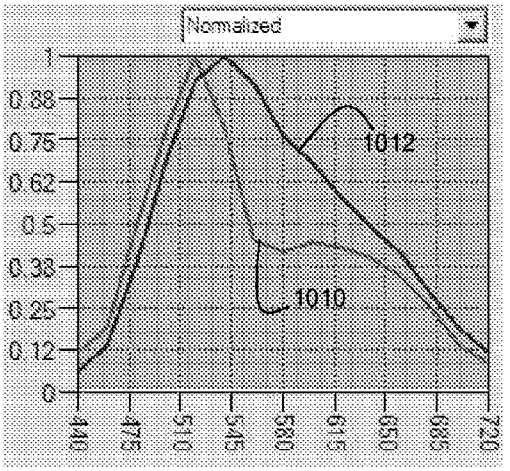

FIGURE 10B

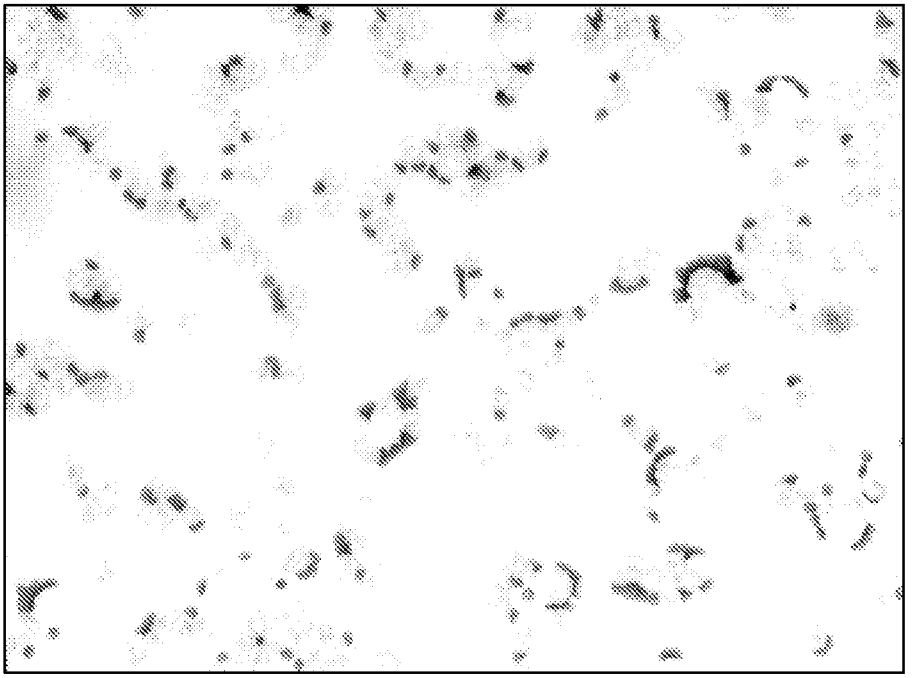

FIGURE 10C

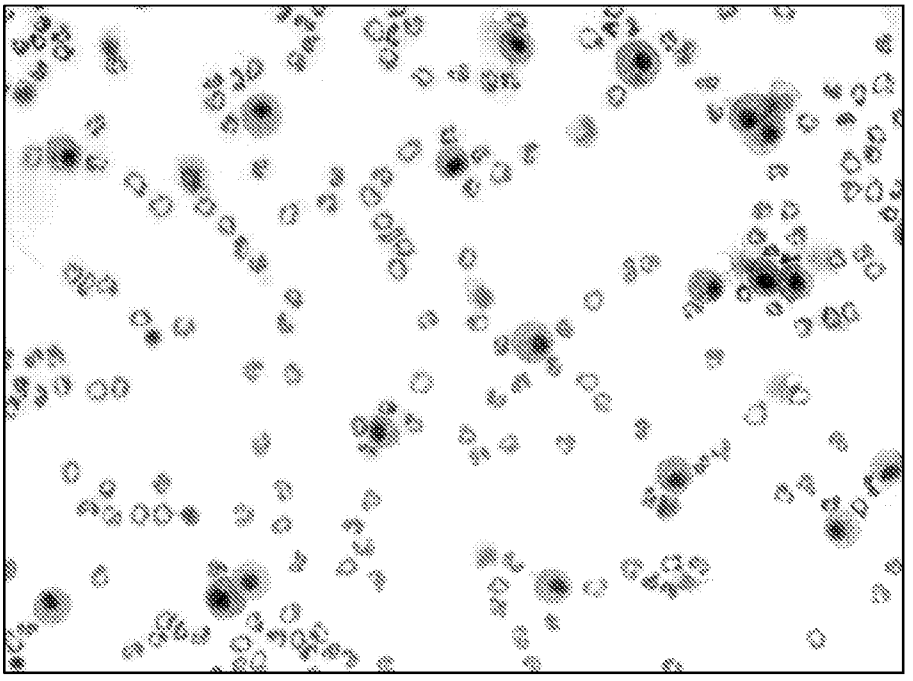

FIGURE 10D

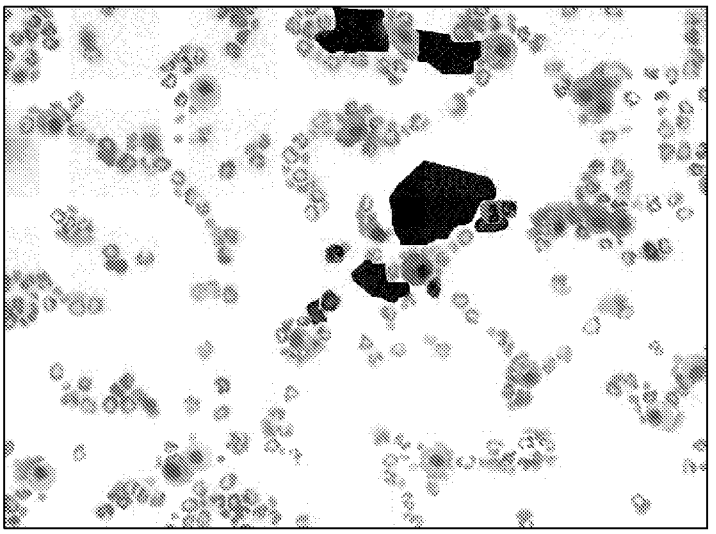

FIGURE 10E

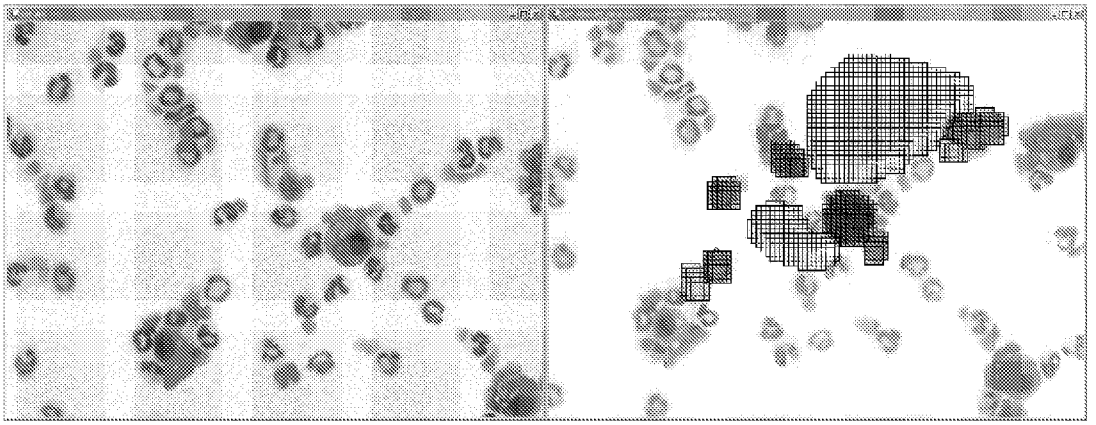

FIGURE 10F

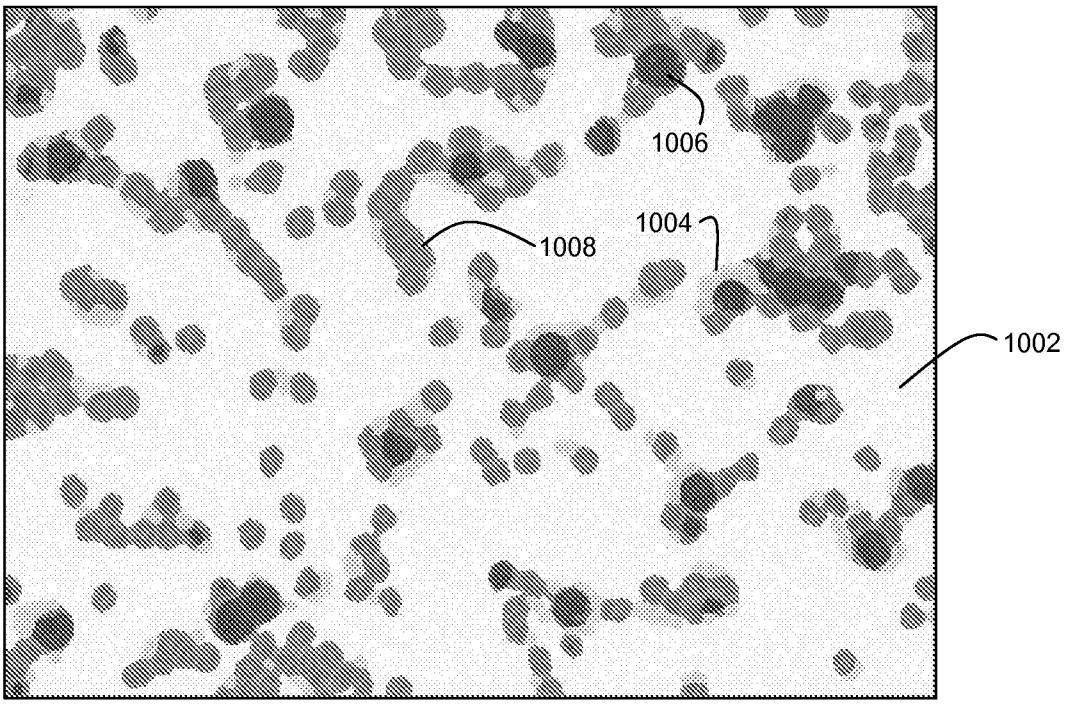

FIGURE 10G

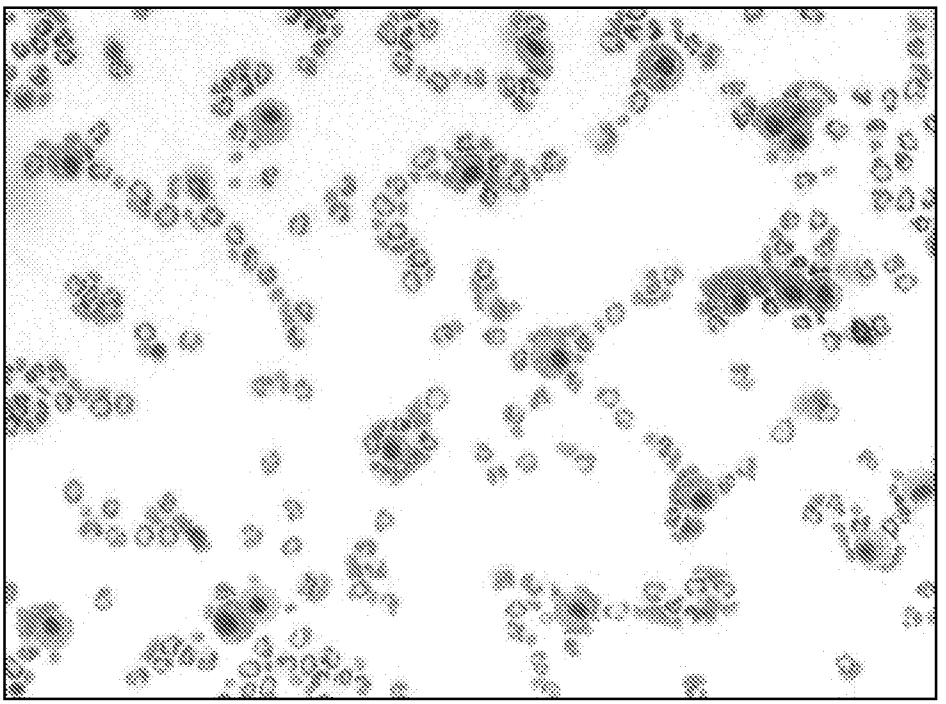

FIGURE 10H

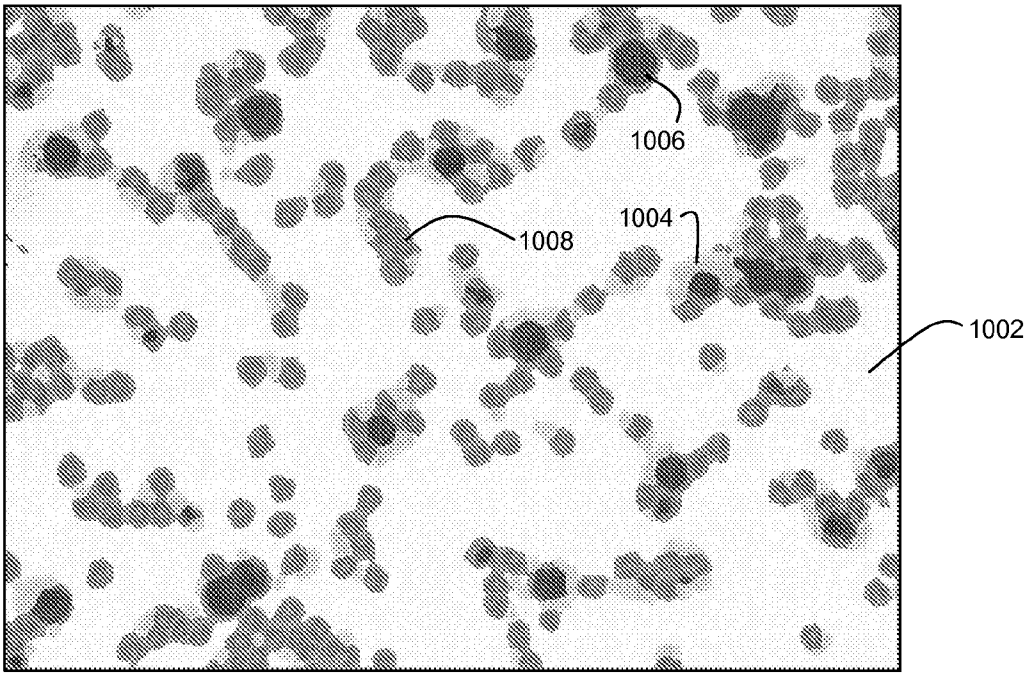

FIGURE 10I

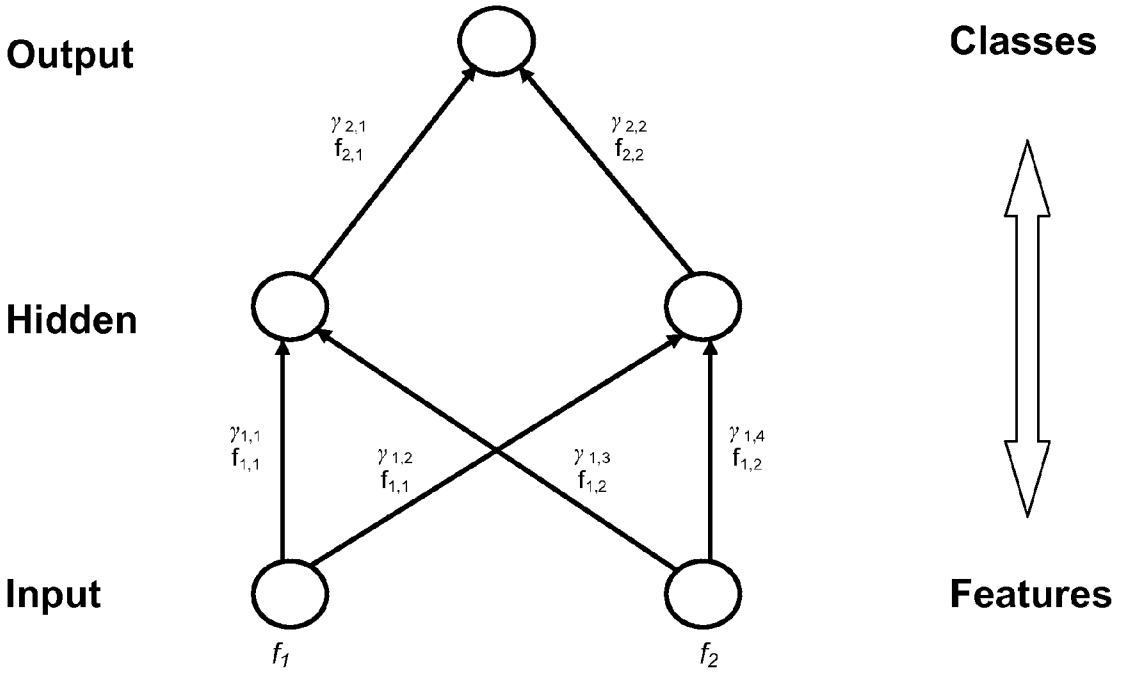
Output
Hidden
Input
Classes
Features
$\gamma_{2,1}$ $f_{2,1}$
$\gamma_{2,2}$ $f_{2,2}$
$\gamma_{1,1}$ $f_{1,1}$
$\gamma_{1,2}$ $f_{1,1}$
$\gamma_{1,3}$ $f_{1,2}$
$\gamma_{1,4}$ $f_{1,2}$
f_1
f_2

FIGURE 11

CLASSIFYING IMAGE FEATURES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of and claims priority to U.S. application Ser. No. 13/600,351, filed on Aug. 31, 2012, which is a continuation of U.S. application Ser. No. 13/091,492, filed on Apr. 21, 2011, now U.S. Pat. No. 8,280,140, which is a continuation of U.S. application Ser. No. 12/477,330, filed on Jun. 3, 2009, now U.S. Pat. No. 7,953,264, which is a continuation of U.S. application Ser. No. 11/342,272, filed on Jan. 27, 2006, now U.S. Pat. No. 7,555,155, which claims priority to U.S. Provisional Patent Application No. 60/647,729 entitled "METHOD FOR CLASSIFYING LABELED PATHOLOGY AND CYTOLOGY TISSUE SECTIONS" by Richard Levenson and Clifford C. Hoyt, filed on Jan. 27, 2005. The contents of the prior applications are incorporated herein by reference in their entirety.

TECHNICAL FIELD

[0002] This invention relates to classifying tissue samples.

BACKGROUND

[0003] Chromogenic staining techniques have been developed empirically to impart visual contrast to various elements within tissue samples. Staining techniques and protocols can produce mixtures of dyes in different tissue elements, and human observers, using microscopes and other imaging devices, have learned to distinguish these staining patterns as typical for particular elements. Modern targeted staining methods, which can be specific to chemical moieties and/or molecular structural arrangements, can produce stained tissues in which two or more chromogenic or fluorescent stains apparently overlap spatially. In fact, the perceived overlap can result because the multiple stains truly are bound within a common structure in the sample, or because, due to the method of preparation, a structure within the sample containing one stain overlaps with a second structure containing a different stain. In either case, it may be difficult to distinguish the presence and relative distribution of the multiple stains and the structures to which they are bound, especially when the stains employed have similar spectral absorption and/or emission characteristics.

[0004] In fields such as pathology and cytology in which staining and inspection of tissue samples occurs frequently, the stained samples are often classified according to one or more criteria by human researchers performing visual inspection of the samples using a microscope or other imaging device. For example, a sample can be stained with multiple dyes in order to highlight differences in particular organelles, structures, or molecular targets among cells in the sample. Samples containing different types of cells can be treated with different dyes in order to visually distinguish the number, spatial distribution, and morphology of the cell types. The samples can then be classified according to one or more criteria such as the presence of different types of chemical or biological structures therein. A wide variety of staining protocols have been developed in order to provide different types of classification information for particular classes of samples.

[0005] As an alternative to the sometimes tedious procedure of manual inspection and classification of tissue samples, machine-vision methods can be employed in an effort to automate the process of sample classification.

SUMMARY

[0006] In general, in a first aspect, the invention features a method that includes classifying different parts of a sample into respective classes based on an image stack that includes one or more images. For example, the sample can be a tissue section.

[0007] Embodiments of the method can include any of the following features.

[0008] The method can further include decomposing a set of spectral images of a sample into an unmixed image set, where each member of the unmixed image set corresponds to a spectral contribution from a different component in the sample, and where the images in the image stack used for classification include one or more of the unmixed images. For example, the images in the image stack used for classification can include some or all of the unmixed images.

[0009] Classifying can include: (i) positioning a sampling window within the image stack to select a portion of the image stack for classification, where the selected portion includes multiple pixels; (ii) classifying the selected portion into one of several classes, where each of the pixels in the selected portion are provisionally classified as having the same class as that of the selected portion; (iii) translating the sampling window to select a second portion of the image stack for classification and classifying the second portion into one of several classes, where each of the pixels in the second portion are provisionally classified as having same class as that of the second portion; (iv) repeating the translating and classifying for the additional portions of the image stack until at least some of the pixels in the image stack have been provisionally classified multiple times as part of different portions selected by the sampling window; and (v) classifying each of at least some of the pixels that have been provisionally classified multiple times into one of the several classes based on their multiple provisional classifications. The different portions selected by the sampling window can include the same number of pixels, and at least some of the different portions selected by the sampling window can overlap with one another. The provisional classifications of each pixel can be expressed as a histogram indicating the number of times the pixel was provisionally classified in each class, and the final classification of each pixel can correspond to the class to which it was most frequently provisionally classified. The number of times at least some of the pixels are provisionally classified can be more than two and no larger than the number of pixels in the sampling window. For example, the number of times at least some of the pixels are provisionally classified can equal the number of pixels in the sampling window. Additionally, the image stack can include only one image.

[0010] The image stack can include more than three spectral images, and the classification can include classifying different regions of the image stack into respective classes based on the set of spectral images, where each region includes multiple pixels so that each classification involves both spectral and spatial information.

[0011] The method can further include generating a composite image based on a set of spectral images of the sample, where the spatial intensities of two or more different spectral images in the set are weighted differently and combined to produce the composite image, and where the one or more images in the image stack include the composite image. For example, the set of spectral images can include n images, and the one or more images in the image stack used for classification can include fewer than n images. The composite image

can be generated by weighting the spatial intensities of the two or more different spectral images in the set according to a function that changes monotonically with a spectral wavelength. The weighting function can be a ramp function that varies linearly with spectral wavelength. Alternatively, the spatial intensities of the two or more different spectral images can be weighted according to a function that changes non-monotonically with a spectral wavelength. For example, the weighting function can include a first portion that changes monotonically with the spectral wavelength and a second portion that changes monotonically with the spectral wavelength, where the slopes of the first and second portions of the weighting function have opposite signs (e.g., the weighting function can be a Gaussian function). The weighting function can be selected to enhance a contrast between features contributed to the composite image from the two or more different spectral images. Further, the one or more images in the image stack can include two or more composite images.

[0012] In any of the methods, a neural network can be used for the classifying. Classifying different regions of the sample into the different classes can include identifying selected regions of the image stack that correspond to each of the individual classes, training the neural network to recognize the classes based on the selected regions, and applying the trained neural network to the additional regions of the image stack. The input into the neural network can be a feature vector having one or more elements based on calculating at least one spatial gray level dependency matrix. Alternatively, or in addition, the input into the neural network can be a feature vector having one or more elements based on calculating a two-dimensional Fourier transform.

[0013] In certain embodiments, the one or more images in the image stack can include one or more spectral images. The spectral images can be images of sample emission according to different spectral indices for the emission, for example. Alternatively, the spectral images can be images of sample emission according to different spectral indices for illumination of the sample causing the emission. Further, the input information for the classifying can include both spectral and spatial information. The sample can include components having different absorption and emission spectra. In addition, a number of classes into which regions of the sample are classified can be equal to a number of distinct spectral contributors in the sample. For example, the distinct spectral contributors can be chemical dyes or fluorescent labels.

[0014] In certain embodiments, the image stack can include an RGB image.

[0015] Also, in certain embodiments, one can further include generating an output image showing the classified regions of the sample. Additionally, any of the methods can further include obtaining the one or more images in the image stack. The images can be obtained, for example, by measuring light transmitted through, or reflected from, the sample. The images can also be obtained by measuring fluorescence emission from the sample.

[0016] In general, in another aspect, the invention features a method that includes: (i) positioning a sampling window within an image stack to select a portion of the image stack for classification, where the image stack includes one or more images and the selected portion includes multiple pixels; (ii) classifying the selected portion into one of several classes, where each of the pixels in the selected portion are provisionally classified as having the same class as that of the selected portion; (iii) translating the sampling window to select a second portion of the image stack for classification and classifying the second portion into one of several classes, where each of the pixels in the second portion are provisionally classified as having same class as that of the second portion; (iv) repeating the translating and classifying for the additional portions of the image stack until at least some of the pixels in the image stack have been provisionally classified multiple times as part of different portions selected by the sampling window; and (v) classifying each of at least some of the pixels that have been provisionally classified multiple times into one of the several classes based on their multiple provisional classifications.

[0017] Embodiments of the method can include any of the foregoing aspects or features of other methods that are suitable for this method.

[0018] In general, in another aspect, the invention features apparatus that includes a computer readable medium storing a program that causes a processor to carry out any of the foregoing methods.

[0019] In general, in another aspect, the invention features apparatus that includes a means for obtaining one or more images of a sample, and an electronic processor for analyzing an image stack based on the obtained images and configured to classify different parts of the sample into respective classes based on the image stack as set forth in any of the foregoing methods.

[0020] Embodiments of the apparatus can include any of the following features.

[0021] The means for obtaining the one or more images of the sample can include means for obtaining spectrally-resolved emission images from the sample. The means for obtaining the one or more images of the sample can include means for obtaining images from the sample corresponding to different spectral illuminations of the sample.

[0022] In general, in another aspect, the invention features apparatus that includes an optical system for obtaining one or more spectral images of a sample, and an electronic processor for analyzing an image stack based on the obtained spectral images and configured to classify different parts of the sample into respective classes based on the image stack as set forth in any of the foregoing methods.

[0023] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict between documents incorporated herein by reference and the present specification, the present specification will control.

[0024] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

[0025] FIG. **1** is a schematic diagram of a system for acquiring spectral images of a sample, and for classifying the sample.

[0026] FIG. **2** is a flow chart showing steps involved in classifying a sample.

[0027] FIG. **3** is a flow chart showing steps involved in training a neural network to perform sample classification.

[0028] FIG. **4** is a schematic diagram showing a region of interest selected for a particular class.

[0029] FIG. **5** is a schematic diagram showing a partitioning of a spatial Fourier transform of a sample image in frequency space into a set of smaller regions.

[0030] FIG. **6** is a flow chart showing steps involved in optimizing a trained neural network.

[0031] FIG. **7** is a flow chart showing steps involved in classifying a sample with a trained neural network.

[0032] FIG. **8** is a schematic diagram showing a region of a sample images selected for classification.

[0033] FIG. **9** shows a calculation of a spatial gray level dependency matrix.

[0034] FIGS. **10**A-**10**I show an example of a classification technique disclosed herein being applied to data for a real sample.

[0035] FIG. **11** is a schematic diagram of a portion of a neural network.

[0036] Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

Overview

[0037] The methods and systems disclosed herein can be used to classify a wide variety of biological and other samples according to spectral and/or structural features appearing on images of the samples. The classification methods include at least some steps that are performed in an automated manner using various machine-vision algorithms and techniques. A set of images of a sample is acquired, and can be transformed prior to submission to an automated classifier. Transformation of the image set can include mathematical transformations such as conversion from intensities to optical densities, spectral unmixing operations, calculation of composite images, and forming a classification data set that may include only a subset of available sample images. The classification data set is then submitted to a machine-based classifier, which can be a neural network or another type of classifier. Image pixels can be classified multiple times, and a final classification performed based on the distribution of the multiple classifications for each pixel. Images illustrating differently-classified regions of the sample can be displayed for a system operator. Classification information can also be used to as an input to direct automated processes such as laser-capture microdissection, or in other image-guided procedures.

[0038] The classification methods are mathematical and so are general in scope, and can be applied wherever classification is desired, regardless of the apparatus or method used to obtain the images, or the nature of the sample. The classification methods can be used to classify a wide variety of samples, including samples stained with one or more absorbing stains, and samples that include fluorescent labels. Fluorescent labels can include chemical labels that are introduced into a sample from an external source; alternatively, the labels can be intrinsic to the sample (e.g., endogenous autofluorescence or fluorescent proteins such as green fluorescent protein and red fluorescent protein). The classification methods can also be used to classify samples containing various luminescent species and structures. The images may be obtained in the visible, infrared, or ultraviolet range. The classification methods are not limited to use with images of sample absorption or sample emission, but can also be used to classify images that utilize a wide variety of measurement or contrast mechanisms to visualize a sample, including but not limited to polarized light, sample birefringence, elastic or inelastic light scattering, or fluorescence lifetime. The classification methods can also be used to classify samples that are imaged with non-optical means such as x-ray transmission or scatter, magnetic resonance, neutron scatter, or positron emission. In short, the classification methods may be used to classify sample regions in any setting where classification of an image is desired. Moreover, the images may be images other than microscopic images. For example, the images can be macroscopic images captured in remote sensing applications. Such images can be detected optically or through other means, as described above.

[0039] As used herein, the term "classifying" refers to identifying different regions of an image of a sample that share a set of common characteristics, wherein at least some of the steps in the procedure are performed in an automated fashion by electronic components. The set of common characteristics can include signal strength, shape, spectral and textural features, for example. The identification of such regions in a sample image effectively identifies the corresponding regions in the sample as sharing a set of common features, and more generally that the sample region is of a specific known state or type based on its expression of these features. At least some of the steps in the classification procedure are performed in automated fashion by electronic components. For example, in many embodiments, steps that include spectral unmixing of images, generating composite images, and classifying regions of images into one or more classes are performed by electronic components. However, some operator intervention may occur in other steps. In particular, in some embodiments, steps such as the selection of reference regions corresponding to various classes for training a machine-based classifier may be performed manually by a system operator.

[0040] In certain embodiments, spectral images of a sample are "unmixed" into images that each correspond to a spectral index of a respective constituent of the sample. These unmixed images can then by processed by the classifier. The use of the unmixed images as the input into the classifier may improve the efficiency and/or accuracy of the classification.

[0041] In certain embodiments, one or more composite images can be generated from spectral images, prior to classification. As explained in more detail later, composite images generally include "flattened" spectral information; that is, composite images contain spectral information that is encoded as variations in a spatial intensity image of a sample. The use of the composite image as an input into the classifier may improve the efficiency and/or accuracy of the classification.

[0042] In certain embodiments, the classification may involve the use of a sampling window to initially classify the pixels in the window, followed by subsequent translations of the sampling window to make further classifications. The translations are smaller than a dimension of the window, so that pixels are classified multiple times. A final classification of each pixel is then based on the statistical distribution of the initial classifications. The technique enables the use of sampling window large enough to recognize spatial features indicative of a specific class, while still providing fine resolution because of the smaller-scale translations.

[0043] In general, the classification methods disclosed herein can be used to classify features in spectral image sets, including color (RGB) images of a sample; or the methods can be used for sample classification where sample images contain no spectral information (i.e., gray scale or monochrome images).

Apparatus for Obtaining Images and Subsequent Classification

[0044] FIG. **1** is a schematic diagram showing a system **100** for acquiring multiple spectrally resolved images of a sample, and for classifying the sample. A light source **102** provides light **122** to light conditioning optics **104**. Light **122** can be incoherent light, such as light generated from a filament source for example, or light **122** can be coherent light, such as light generated by a laser. Light **122** can be either continuous-wave (CW) or time-gated (i.e., pulsed) light. Further, light **122** can be provided in a selected portion of the electromagnetic spectrum. For example, light **122** can have a central wavelength and/or a distribution of wavelengths that falls within the ultraviolet, visible, infrared, or other regions of the spectrum.

[0045] Light conditioning optics **104** can be configured to transform light **122** in a number of ways. For example, light conditioning optics **104** can spectrally filter light **122** to provide output light in a selected wavelength region of the spectrum. Alternatively, or in addition, light conditioning optics can adjust the spatial distribution of light **122** and the temporal properties of light **122**. Incident light **124** is generated from light **122** by the action of the elements of light conditioning optics **104**.

[0046] Incident light **124** is directed to be incident on sample **108** mounted on illumination stage **106**. Stage **106** can provide means to secure sample **108**, such as mounting clips or other fastening devices. Alternatively, stage **106** can include a movable track or belt on which a plurality of samples **108** are affixed. A driver mechanism can be configured to move the track in order to successively translate the plurality of samples, one at a time, through an illumination region on stage **106**, whereon incident light **124** impinges. Stage **106** can further include translation axes and mechanisms for translating sample **108** relative to a fixed position of illumination stage **106**. The translation mechanisms can be manually operated (e.g., threaded rods) or can be automatically movable via electrical actuation (e.g., motorized drivers, piezoelectric actuators).

[0047] In response to incident light **124**, emitted light **126** emerges from sample **108**. Emitted light **126** can be generated in a number of ways. For example, in some embodiments, emitted light **126** corresponds to a portion of incident light **124** transmitted through sample **108**. In other embodiments, emitted light **126** corresponds to a portion of incident light **124** reflected from sample **108**. In yet further embodiments, incident light **124** can be absorbed by sample **108**, and emitted light **126** corresponds to fluorescence emission from sample **108** in response to incident light **124**. In still further embodiments, sample **108** can be luminescent, and may produce emitted light **126** even in the absence of incident light **124**. In some embodiments, emitted light **126** can include light produced via two or more of the foregoing mechanisms.

[0048] In many embodiments, sample **108** is a biological sample such as a tissue slice (e.g., a sample used for pathology, or a cell suspension or smear, as in cytology studies), or living or fixed cells in tissue culture. In some embodiments, sample **108** can be an animal (e.g., a mouse), individual bacteria or other microorganisms, bacterial or other colonies, embryos, oocytes, plants, including seeds or grains, or sample **108** can be a non-biological entity.

[0049] Light collecting optics **110** are positioned to received emitted light **126** from sample **108**. Light collecting optics **110** can be configured to collimate emitted light **126** when light **126** is divergent, for example. Light collecting optics **110** can also be configured to spectrally filter emitted light **126**. Filtering operations can be useful, for example, in order to isolate a portion of emitted light **126** arising via one of the mechanisms discussed above from light arising via other processes. Further, light collecting optics **110** can be configured to modify the spatial and/or temporal properties of emitted light **126** for particular purposes in embodiments. Light collecting optics **110** transform emitted light **126** into output light **128** which is incident on detector **112**.

[0050] Detector **112** includes one or more elements such as CCD sensors configured to detect output light **128**. In embodiments, detector **112** can be configured to measure the spatial and/or temporal and/or spectral properties of light **128**. Detector **112** generates an electrical signal that corresponds to output light **128**, and is communicated via electrical communication line **130** to electronic control system **114**.

[0051] Electronic control system **114** includes a processor **116**, a display device **118**, and a user interface **120**. In addition to receiving signals corresponding to output light **128** detected by detector **112**, control system **114** sends electrical signals to detector **112** to adjust various properties of detector **112**. For example, if detector **112** includes a CCD sensor, control system **114** can send electrical signals to detector **112** to control the exposure time, active area, gain settings, and other properties of the CCD sensor.

[0052] Electronic control system **114** also communicates with light source **102**, light conditioning optics **104**, illumination stage **106**, and light collecting optics **110** via electrical communication lines **132**, **134**, **136**, and **138**, respectively. Control system **114** provides electrical signals to each of these elements of system **100** to adjust various properties of the elements. For example, electrical signals provided to light source **102** can be used to adjust the intensity, wavelength, repetition rate, or other properties of light **122**. Signals provided to light conditioning optics **104** and light collecting optics **110** can include signals for configuring properties of devices that adjust the spatial properties of light (e.g., spatial light modulators) and for configuring spectral filtering devices, for example. Signals provided to illumination stage **106** can provide for positioning of sample **108** relative to stage **106** and/or for moving samples into position for illumination on stage **106**, for example.

[0053] Control system **114** includes a user interface **120** for displaying system properties and parameters, and for displaying captured images of sample **108**. User interface **120** is provided in order to facilitate operator interaction with, and control over, system **100**. Processor **116** includes a storage device for storing image data captured using detector **112**, and also includes computer software that embodies instructions to processor **116** that cause processor **116** to carry out control functions, such as those discussed above for example. Further, the software instructions cause processor **116** to mathematically manipulate the images captured by detector **112** and to carry out the steps of classifying sample **108** according to either or both of the original and the manipulated images. The classification steps are described in more detail subsequently.

[0054] In many embodiments, system **100** is configured to acquire multiple spectral images of sample **108**. The multiple spectral images may correspond to illumination of sample **108** at a variety of selected wavelengths of light, and detecting an intensity of light either transmitted through or reflected by sample **108**. Alternatively, the multiple spectral images may

correspond to illumination of sample **108** with light having similar spectral properties, and collecting multiple images of sample **108**, each image corresponding to a different wavelength of emitted light **126**. Spectral filtering elements in light conditioning optics **104** and light collecting optics **110** are generally used to obtain the spectrally resolved data.

[0055] In some embodiments, images of sample **108** can be collected in sequence, with adjustments to the configuration of optical components (e.g., optical filters) between successive captured images. In other embodiments, multiple images can be captured simultaneously using detection systems configured to detect multiple sample views. For example, detection systems can be configured to project different views of the sample corresponding to different illumination or emission wavelengths onto a detector such as a CCD camera, and the multiple views can be captured simultaneously.

[0056] In some embodiments, light conditioning optics **104** include an adjustable spectral filter element such as a filter wheel or a liquid crystal spectral filter. The filter element can be configured to provide for illumination of sample **108** using different light wavelength bands. Light source **102** can provide light **122** having a broad distribution of spectral wavelength components. A selected region of this broad wavelength distribution is allowed to pass as incident light **124** by the filter element in light conditioning optics **104**, and directed to be incident on sample **108**. An image of light **126** transmitted through sample **108** is recorded by detector **112**. Subsequently, the wavelength of the filter pass-band in light conditioning optics **104** is changed to provide incident light **124** having a different wavelength, and an image of light **126** transmitted through sample **108** (and corresponding to the new wavelength of incident light **124**) is recorded. A similar set of spectrally-resolved images can also be recorded by employing a light source **102** having multiple source elements generating light of different wavelengths, and alternately turning the different source elements on and off to provide incident light **124** having different wavelengths.

[0057] As discussed previously, the emitted light **126** from sample **108** can also correspond to incident light **124** that is reflected from sample **108**. Further, emitted light **126** can correspond to fluorescence emission from sample **108** if the sample includes fluorescent chemical structures. For some samples, emitted light **126** can include contributions from multiple sources (i.e., transmission and fluorescence) and the spectral filtering elements in light conditioning optics **110** can be used to separate these signal contributions.

[0058] In general, both light conditioning optics **104** and light collecting optics **110** include configurable spectral filter elements. Therefore, spectral resolution can be provided either on the excitation side of sample **108** (e.g., via light conditioning optics **104**) or on the emission side of sample **108** (e.g., via light collecting optics **110**), or both. In any case, the result of collecting multiple, spectrally resolved images of sample **108** is an "image stack" where each image in the stack is a two-dimensional image of the sample corresponding to a particular wavelength. Conceptually, the set of images can be visualized as forming a three-dimensional matrix, where two of the matrix dimensions are the spatial length and width of each of the images, and the third matrix dimension is the spectral wavelength (emission or excitation) to which the image corresponds. For this reason, the set of spectrally resolved images can be referred to as a "spectral cube" of images. As used herein, a "pixel" in such a set of images (or image stack or spectral cube), refers to a common spatial location for each of the images. Accordingly, a pixel in a set of images includes a value associated with each image at the spatial location corresponding to the pixel.

[0059] Other arrangements to obtain spectral images which are known in the art may be employed, according to the requirements of the sample at hand.

[0060] While each spectral image described above typically refers to a particular wavelength or range of wavelengths (e.g., a spectral band), more generally, each spectral image can correspond to a spectral index that may include one or more wavelength bands, or some more complex spectral distribution. For example, such an image can be generated by using a spectral comb filter. Generally, the image cube will include several spectral images, for example, 10 or more. However, in some embodiments, the image cube may include fewer images, for example, only two or three spectral images. One such example is an red-green-blue (RGB) color image, in which each pixel includes a value associated with the strength of each of the red, green, and blue colors. Such information may be displayed as a single color image, rather than as a set of separate images; however, the information content is the same as that in the set of images, and therefore we use the expression "spectral images" to refer to both cases.

[0061] In certain embodiments, images used for classification may also include false-color images, and also monochrome or gray scale images.

[0062] Following acquisition of one or more images, sample **108** is classified by system **100** according to the shape, intensity, spectral and/or textural features of the individual image(s). In practice, in some embodiments, images are recorded for multiple samples first, and the classification of the samples is deferred to a later time for expediency.

[0063] Not all of the images of a spectral cube need be analyzed in order to accurately classify the sample to which the cube corresponds. In some embodiments, a classification of sufficiently high accuracy is achieved by examining only a subset of the spectral cube images. Further, in some embodiments, the spectrally resolved images may be spectrally unmixed (i.e., decomposed into a set of images corresponding to a set of spectral eigenstates) before analysis. Some embodiments include additional steps wherein one or more composite images are generated via mathematical combination of multiple images selected from the spectral cube and/or the set of spectrally unmixed images. Classification of a sample can be performed based on the composite images, in addition to or exclusive of the spectral cube images and the spectrally unmixed images.

Spectral Unmixing

[0064] FIG. **2** is a flow chart **200** showing steps involved in classifying a sample. Step **202** includes acquiring a set of one or more images (e.g., a spectral cube) of a sample, as discussed above. Step **204**, which is optional, includes spectrally unmixing some or all of the images in the spectral cube to generate an unmixed set of images (i.e., an "unmixed spectral cube"). Spectral unmixing is a technique that quantitatively separates contributions in an image that arise from spectrally different sources. For example, a sample may contain three different types of structures, each labeled with a different dye. The three different dyes may each have different absorption spectra. Typically, the individual absorption spectra of the dyes are known before they are used, or they can be measured. Images of the specimen under illumination will contain, in the most general case, spectral contributions from each of the

three dyes. A similar situation arises, for example, in samples containing multiple different fluorescence labels, each of which contribute to measured fluorescence emissions.

[0065] Spectral unmixing decomposes one or more images that include contributions from multiple spectral sources into a set of component images (the "unmixed images") that correspond to contributions from each of the spectral entities within the sample. Thus, if the sample includes three different dyes, each specific to a particular structural entity, then an image of the sample can be separated into three unmixed images, each unmixed image reflecting contributions principally from only one of the dyes.

[0066] The unmixing procedure essentially corresponds to decomposing an image into a set of spectral eigenstates. In many embodiments, the eigenstates are known beforehand, as discussed above. In other embodiments, the eigenstates can sometimes be determined using techniques such as principal component analysis. In either case, once the eigenstates have been identified, an image can be decomposed by calculating a set of values, usually as a coefficient matrix, that corresponds to the relative weighting of each of the eigenstates in the overall image. The contributions of each of the individual eigenstates can then be separated out to yield the unmixed image set.

[0067] As an example, a series of two dimensional images having x and y coordinates can be measured for a sample by illuminating the sample at a set of different excitation wavelengths λ_k. As described above, the two dimensional images can be combined to form a three-dimensional image cube I(x,y,k) where the first two indices of the image cube represent coordinate directions, and the third index is a spectral index corresponding to the wavelength of the illumination light. Assuming, for the sake of simplicity, that each of the images of the sample contains spectral contributions from two different spectral sources $F(\lambda_k)$ and $G(\lambda_k)$, then the values in the three-dimensional image cube I(x,y,k) may be given by

$$S(x,y,k)=a(x,y)\cdot F(\lambda_k)+b(x,y)\cdot G(\lambda_k) \quad (1)$$

where λ_k is used to denote a given wavelength (or wavelength band). The functions a(x,y) and b(x,y) describe the spatial abundance of the spectral contributions from the two different spectral sources in the sample.

[0068] According to Equation (1), the net signal any position in the three-dimensional image cube (i.e., at any two-dimensional pixel coordinate, and at a particular illumination wavelength) is the sum of two contributions, weighted by the relative abundance of each. This can be expressed as

$$I(\lambda_k)=aF(\lambda_k)+bG(\lambda_k) \quad (2)$$

[0069] The functions F and G can be termed the "spectral eigenstates" for the system because they correspond to the pure spectra for the spectral sources in the sample, which are combined in varying proportions to produce the measured spectral images of the sample. Thus, the sample spectrum is a weighted superposition corresponding to separate contributions from the two spectral sources.

[0070] If the spectra $F(\lambda_k)$ and $G(\lambda_k)$ are known (or can be deduced), then Equation (2) can be inverted to solve for a and b, provided that spectrum I includes at least two elements (i.e., provided that one has data for at least two wavelengths λ_k). Equation (2) can be rewritten in matrix form as I=EA, so that

$$A=E^{-1}I \quad (3)$$

where A is a column vector with components a and b, and E is a matrix whose columns are the spectral eigenstates, namely [F G].

[0071] Using Equation (3), measured spectral images of a sample can be used to calculate contributions to the images arising purely from source F and purely from source G at particular pixel locations. The process can be repeated for each pixel location on a selected image (i.e., throughout the range of values x and y in I) to produce an image of the sample that includes contributions only from source F, and another image of the sample that includes contributions only from source G.

[0072] In the above discussion, the number of spectral sources is two (i.e., F and G). In general, however, unmixing techniques are not restricted to any particular number of sources. For example, a sample can generally contain m different spectral sources. If the number of wavelengths at which data is collected is n—that is, k=1 . . . n—then matrix E is an n×m matrix instead of an n×2 matrix, as in the above discussion. The unmixing algorithm can then be employed in the same manner as described above to isolate specific contributions at each pixel location in an image from each of the m spectral eigenstates.

[0073] One factor which can limit the ability of the algorithm to distinguish between contributions from different spectral eigenstates is the degree of spectral distinction between the eigenstates. The correlation between two spectra, such as two spectral eigenstates I_1 and I_2, can be described by a spectral angle θ where

$$\theta=\cos^{-1}\left[\frac{I_1\cdot I_2}{|I_1||I_2|}\right] \quad (4)$$

[0074] Sets of spectra for which θ is small for two members are not as easily separated into their components. Physically, the reason for this is easily understood: if two spectra are only marginally different, it is harder to determine the relative abundance of each.

[0075] A number of techniques can be used to measure or estimate the pure spectra of the spectral sources F and G (and other spectral sources, where the sample includes more than two). In general, any method that yields spectral eigenstates of sufficient accuracy can be used. Some samples can contain spectral sources such as dyes, fluorescence labels, or other chemical moieties for which there are known spectra available in published reference materials. Alternatively, it may be possible to directly measure the spectra of source components using one or more measurement systems. In some samples, a particular region of the sample may be known to include only one particular spectral source, and the spectrum of that source can be extracted from measurements taken on only the identified region of the sample.

[0076] Various data analysis techniques can also be used for determining component spectra for spectral unmixing, such as principal component analysis (PCA), which identifies the most orthogonal spectral eigenvectors from an image cube and yields score images showing the weighting of each eigenvector throughout the image. This may be done in combination with other mathematical processing, and there are other known techniques for identifying low-dimensionality spectral vectors, such as projection pursuit, a technique described, for example, in L. Jimenez and D. Landgrebe, "Hyperspectral Data Analysis and Feature Reduction Via Projection Pursuit", *IEEE Transactions on Geoscience and Remote Sensing*, Vol. 37, No. 6, pp. 2653-2667, November 1999, the entire contents of which are incorporated herein by

reference. Other techniques include independent component analysis (ICA) and end-member detection algorithms, for example.

[0077] These techniques are typically not well-suited to the applications in the life sciences. For example, some techniques are optimized for spectral imaging data sets that contain spectra with dense spectral shapes and well-defined narrow peaks. In some techniques the spectral ranges are large compared to the individual spectral features and peaks that are used for analysis. The presence of peaks, or the ratio of peaks may be then used to classify "end-members" to be separated. Unfortunately, the components in biological samples typically do not have such well-defined, narrow peaks.

[0078] Some of these techniques generate images related to spectra that are present in a pure form somewhere within the original image cube. In many cases in the life sciences, signal spectra present in the image cube are mixtures of components. If the component of interest is not in a pure form somewhere in the original image cube, then it is unlikely that these techniques will generate an image that accurately represents the abundance of the component of interest.

[0079] There are some techniques, sometimes called "convex-hull" algorithms, that estimate what the true end-members are even if they do not exist in a pure form in the image, but the effectiveness is dependent on how close signal spectra in the image cube are to the end-members.

[0080] One technique that can be used to extract spectral eigenstates (or representations thereof) without a priori knowledge of all of the eigenstates involves considering the signal spectrum $I(\lambda_k)$ for a given pixel, and subtracting from it the maximum amount of a first spectral source $F(\lambda_k)$ while leaving the remaining signal that is positive definite in all spectral channels. That is, one defines a so-called "remainder spectrum" $U_a(\lambda_k)$ for each pixel as

$$U_a(\lambda_k)=I(\lambda_k)-aF(\lambda_k) \quad (5)$$

and then selects the largest value of the parameter a consistent with $U_a(\lambda_k)$ having a non-negative value in every spectral channel. The resulting spectrum $U_a(\lambda_k)$ is then used as the signal spectrum, expunged of contributions due to first spectral source F. One may also make the determination of parameter a based not on strict non-negative criterion listed above, but on some related criteria that incorporates a small negative distribution, to account for considerations such as shot noise or detector noise in a measurement system. Additional examples of optimization criteria for removing the maximal amount of spectral source F include using different error functions.

[0081] Alternatively, one may seek to extract a contribution to a measured spectrum that is due to second spectral source G. In analogy with Equation (5), the remainder spectrum can be calculated for each pixel as

$$U_b(\lambda_k)=I(\lambda_k)-bG(\lambda_k) \quad (6)$$

where one selects the largest value of the parameter b consistent with $U_b(\lambda_k)$ having a non-negative value in every spectral channel.

[0082] The remainder technique can be expanded to cases where the spectra for one or more additional components of the sample are known, and one wants to remove their contributions to the signal. In such cases, the remainder spectrum is written to subtract a contribution of each such component from the observed signal based on the additional spectra and consistent with a positive remainder in each spectral channel.

[0083] Additional spectral unmixing techniques are described in PCT Patent Publication No. WO2005/040769 entitled "SPECTRAL IMAGING OF BIOLOGICAL SAMPLES" by Richard Levenson et al., the contents of which are incorporated herein by reference.

[0084] In order for the spectral unmixing techniques disclosed herein to effectively separate contributions in sample images that are due to different spectral eigenstates, Equation (1) should be at least approximately correct. That is, the measured spectral data should be approximately described as a linear superposition of weighted eigenstates. This approximation holds for many samples and spectral measurement techniques, especially darkfield measurement techniques. For example, sample images arising from fluorescent or luminescent chemical labels within the sample typically satisfy the linearity assumption. In some cases however, such as for some brightfield measurement techniques, the linearity approximation may not be satisfied. For example, when images are captured that arise from illumination light that is transmitted through a sample that includes light-absorbing components, the linearity assumption in Equation (1) may not be correct. Instead, the intensity of the measured light may be reduced with an exponential dependence on the concentration of the light-absorbing components. In such cases, transformation of the images may first be necessary before unmixing techniques can be used. As an example, for sample images measured in a transmission mode, the measured image intensities can be transformed into optical densities (e.g., by applying a logarithmic function) in order to apply linear unmixing techniques. Optical density techniques are further described, for example, in U.S. application Ser. No. 10/226,592 (Publication No. US 2003/0081204 A1) entitled "SPECTRAL IMAGING" by Paul J. Cronin and Peter J. Miller, filed Aug. 23, 2002, the entire contents of which are incorporated herein by reference.

[0085] Spectral unmixing operations (e.g., matrix inversion techniques and remainder techniques) and image data transformation operations (e.g., converting measured image intensities to optical densities, where appropriate) can be performed by electronic control system **114** via processor **116**, for example. These operations can include manual intervention and configuration steps performed by a system operator, or system **100** can be configured to perform these operations in an automated manner.

Composite Images

[0086] Application of the unmixing techniques discussed above provides a set of unmixed images from a multi-spectral data set. Returning now to FIG. **2**, in a second optional step in flow chart **200**, step **206** includes generating one or more composite images using the spectral cube images and/or unmixed spectral cube images. Composite images are generated as a means to "flatten" or compress spectral information into a two-dimensional grayscale image. In other words, in terms of a 3D spectral matrix of image data, generating a composite image corresponds roughly to compressing or packing the information from two or more layers into a single layer. Since both spectral cube and unmixed spectral cube image data can be used, the technique can conceptually include packing multiple layers from different spectral cubes into a single layer.

[0087] As an example, consider a 3D spectral cube of images, where each image has width x, height y, and an index k that corresponds to a wavelength λ_k. If there are a total of N

different images in the cube (i.e., data recorded at N different wavelengths) then the spectral cube I can be represented, as described previously, as a matrix I(x,y,k). Compressing spectral information from two or more images in the spectral cube to create a composite image C is equivalent to adding the image layers together. In some embodiments, prior to adding the layers together, each layer is scaled according to a weighting function $f(k)$. The spectral compression operation is then performed according to

$$C(x, y) = \sum_{k=m}^{n} f(k) \cdot I(x, y, k) \tag{7}$$

which yields composite image C(x,y) from layers m through n of the spectral image cube. The weighting function $f(k)$ is generally chosen to emphasize different spectral features in the composite image; that is, to create contrast between features arising from the different layers of the spectral cube that contribute to the overall intensity distribution in the composite image.

[0088] A wide variety of weighting functions can be chosen in order to produce the desired contrast. In general, in some embodiments, a monotonically increasing or decreasing function is chosen for $f(k)$, such as a linear ramp function or a sigmoidal function. In other embodiments, $f(i)$ can be a dual ramp function (i.e., decreasing to a point and then increasing, or increasing to a point and then decreasing) or another function, such as one or more Gaussian functions. The weight function can generally be selected as desired, and can be applied to a batch series of samples, or can be selected individually for each sample prior to classification. System **100** can include a storage medium to store weighting functions for particular types of samples, so that a weighting function appropriate for a sample undergoing classification can be recalled as needed.

[0089] Step **208** includes selecting a set of images to be classified. In general, any or all of the images from the spectral image cube, the unmixed spectral image cube (if calculated), and the composite images (if calculated) can be selected for classification analysis. In some embodiments, for example, classification of a sample to a high degree of accuracy can be achieved using a composite image and a small subset of either spectral cube images or unmixed spectral cube images. This has the advantage that the overall set of data upon which a classification algorithm operates is greatly reduced, increasing the speed with which the classification of the sample is complete.

[0090] In some other embodiments, images from the unmixed spectral cube can be used for sample classification. The images can be delivered to a classification algorithm, and may be accompanied (although not always) by one or more composite images.

[0091] In some embodiments, more than three spectral images can be used for classification of a sample. The images can be taken from either the spectral image cube or, if calculated, an unmixed spectral image cube. This technique can be particularly advantageous when the sample includes more than three distinct spectral contributors. For example, the sample can contain four different stains or dyes, or four different fluorescent labels.

[0092] In other embodiments, color RGB images or single plane images can be used for classification of a sample. Single plane images may be narrow band or panchromatic.

Classification

[0093] In general, the classifier is a mechanism or rule-set to assign a sample to one of several output classes, and it can be any linear or nonlinear classifier. Linear classifiers include least-squares distance, Mahalanobis distance, and others. These may be used, but the classifier is preferably a machine-learning algorithm such as a neural network, genetic algorithm, or support vector machine. However, a neural network is often preferred, and will be used as the example throughout the subsequent discussion.

[0094] The neural network is generally applied to one or more areas, each of which typically corresponds to several pixels (e.g., a 2×2 set of pixels, or a 16×16 set of pixels, etc.) in the image stack (which, as described above, may include one or more images). When there is more than one image in the image stack, each pixel will include a value associated for each of the images. The values for all of the pixels in a given area being classified form the basis of the input information that can potentially be applied to the neural network. Because each area includes several pixels, the input information available to the neural network includes both spatial and spectral information when the image stack includes a composite image and/or multiple spectral images.

[0095] The neural network has one or more input nodes, by which it receives information about the region to be classified. An input is termed a "feature vector," where each element of the feature vector corresponds to a specific input node of the neural network. The elements of the feature vector are functions of the signal values at one or more pixels in the area being classified. Examples of suitable functions for producing the feature vector are described further below.

[0096] The neural network will also have several output nodes, each corresponding to a class to which the area may be designated. When the feature vector for a given area is applied to the neural network, values for the output nodes correspond to the degree to which the area should be assigned to a given class. Preferably, the neural network is trained so that the output node values are binary, with only one output node yielding a non-zero value (and indicating the class to which the area should be assigned) for any given feature vector.

[0097] As described in further detail below, the neural network is trained and can be further optimized to reduce the number of input nodes necessary for efficient and accurate classification. In many embodiments, the use of unmixed images and/or one or more composite images can lead to a reduction in the number of input nodes, and therefore greater efficiency when classifying the regions of an unknown sample. The topology of the neural network employed in some embodiments is bipolar, although binary and other neural network types can also be used effectively. The network is trained using a back propagation method, with momentum included in the training algorithm. The activation function of the network in some embodiments is a bipolar sigmoidal function; other activation functions can also be used.

[0098] In embodiments, the networks can commonly include 0, 1, or 2 hidden layers, which are the layers between a first layer having the input nodes and the last layer having the output nodes, although additional hidden layers are possible. Anywhere from 1-15 nodes per hidden layer and are common, though again additional nodes can be used. The

input layer of the network uses spatial and spectral texture features identified on sample images as input. The output layer includes a number of output nodes equal to the number of identified classes N_c.

[0099] FIG. **11** is a schematic diagram showing an example of a neural network that can be used in the classification methods disclosed herein. The network includes an input layer, one hidden layer, and an output layer. Inputs to the neural network are feature vectors f_m, and coupling strengths between nodes are given by $\gamma_{k,l}$ values. The outputs from the neural network are the classes associated with an image or image stack.

[0100] Typical topological parameters for networks used in the processing of tissue sample images include one hidden layer with 5 nodes, a learning parameter of 0.2, and a momentum factor of 0.5. The structure of neural networks are described, for example, in Christopher M. Bishop, "Neural Networks for Pattern Recognition", Oxford University Press, 1995.

[0101] Referring again to FIG. **2**, after selecting the set of images according to which the sample will be classified (the "classification image set"), step **210** includes training the classifier using images from the classification image set.

[0102] The neural network is trained when a new type of sample is presented for classification analysis. A system operator can be provided with a choice to re-train the existing network for a particular sample via display device **118**, for example. The procedure for training the neural network is discussed in greater detail subsequently.

[0103] After the neural network-based classifier is trained, step **212** includes submitting the classification image set to the classifier. The classifier generally classifies portions of the sample according to textural and spectral features present on images of the sample in the classification image set. The details of the steps involved in the classification routine are presented later.

[0104] Finally, step **214** includes generating classification output for the sample. The classification output can include, for example, one or more images constructed to show contrast between differently classified regions of the sample. Alternatively, or in addition, the classification output can include warning sounds or messages to indicate the presence or absence of particular elements (i.e., stained or labeled structures) in the sample. The output can also include numeric data indicating the types of regions present in the sample, their relative abundance, and other numerical parameters describing the sample.

Training the Neural Network

[0105] FIG. **3** is a flow chart **300** that includes steps for training the neural network classifier. A first step **302** includes determining a number of classes N_c to search for in the image stack. In many embodiments, the number of classes is selected to correspond to the number of different states that are expected or sought within the sample. This may be greater than the number of spectral planes in the image set, or it may be fewer. For example, a sample may be stained with three different dyes or labeled with three different fluorescent labels. In such a sample, one may seek to identify three different classes N_c, or two, or five, according to the structure and nature of the sample. The classifier is capable of resolving more classes N_c than the number of spectral planes, based on other aspects of the sample such as signal strength, shape, and texture.

[0106] The second step **304** includes selecting at least one training region of interest (ROI) for every class on one of the sample images (the pixel spatial coordinates (x,y) of the ROIs for each of the classes are assumed to be the same from one image to the next). The training ROIs are known to correspond to respective classes and provide a reference for the neural network algorithm to allow it to determine particular spectral and spatial features which are common to each of the classes in order to assist in classification decisions. In some embodiments, for example, the selection of ROIs occurs dynamically via interaction with a system operator through display device **118** and user interface **120**.

[0107] The third step **306** includes selecting a sub-sampling window size. A sub-sampling window is used to examine each of the selected ROIs at a finer level of detail. In many embodiments, the sub-sampling window size is chosen to be smaller than the mean length and width of all of the ROIs, but larger than a single pixel within the ROI. The sub-sampling window width is also frequently chosen to have both width and length that are multiples of 2, because Fourier methods that operate on sub-sampled regions of the ROIs can take advantage of FFT algorithms if the variable space is a multiple of 2. In embodiments, typical sub-sampling window sizes include 4×4 pixels, 8×8 pixels, 16×16 pixels, and 32×32 pixels, although a wide variety of window sizes, including window sizes not listed explicitly herein, are also possible. Moreover, while the presently described embodiments presume that the data for each image is represented with respect to a two-dimensional grid of squares, other embodiments may include a different representation of data and corresponding window and ROI dimensions. For example, the data may represented on a hexagonal grid, or some other shape.

[0108] The next series of steps involve operations conducted on each of the identified classes. Each of the classes is analyzed in turn. Step **308** includes selecting a ROI corresponding to a currently selected class. Step **310** includes examination of the ROI by sub-sampling the ROI with the selected sub-sampling window. FIG. **4** shows the sub-sampling process in greater detail. A chosen ROI **400** is sub-sampled by a sub-sampling window **402** that selects a fraction of the image pixels within ROI **400** for analysis.

[0109] Returning to FIG. **3**, step **312** includes calculating and storing a feature vector for each of the sub-sampled regions of the ROI. The feature vector includes as elements a set of numbers calculated from the sub-sampled pixels of the ROI. Each of the calculated feature vectors correspond to a feature vector that would, for a properly trained neural network, output a classification corresponding to the selected class. The elements of the feature vector generally correspond to particular texture analysis features which provide a basis for classification of regions within an image of the sample.

[0110] Many different numerical quantities can be calculated in order to provide a sufficiently distinguishable description of the ROI. For example, in some embodiments, the feature vector corresponding to a selected ROI for a particular class can include 10 different calculations for each of the images in the image stack, thereby resulting in vector with $10N_i$ elements, where N_i, is the number of images in the image stack. The first four of the ten calculations can be texture analysis features obtained from spatial gray level dependency matrices (SGLDMs), which are also referred to as co-occurrence matrices. For example, such matrices are described in R. M. Haralick, K. Shanmugam, and I. Dinstein, "Textural features for image classification", *IEEE Trans.*

Syst., Man, Cybern., vol. SMC-3, pp. 610-621, 1973. A SGLDM is a spatial histogram of an image (or a portion thereof) that quantifies a distribution of gray scale values within the image. SGLDMs can be calculated, for example, from an estimate of the second-order joint conditional probability densities, $s_\theta(i,j|d,\theta)$. Each value of this conditional probability density represents the probability of a pixel having a gray level value i being d pixels away from a pixel having a gray level value j in a direction described by θ. If an image includes N_g gray levels, then an $N_g \times N_g$ matrix $s_\theta(i,j|d,\theta)$ can be created. Optionally, the matrix can be summed over a set of directions θ for a selected distance d. For example, in some embodiments, a single direction θ=0° can be selected. In other embodiments, for example, four directions can be employed: θ=0°, 45°, 90°, and 135°. In general, any number of directions can be selected for analysis of the texture features in a particular ROI.

[0111] In some embodiments, the distance d is fixed at a particular value for analysis. For example, the distance d can be fixed at a value of 1 pixel. In other embodiments, a range of distances can be used, depending upon the nature of the specific texture features. In general, the distance d and the direction θ can be regarded as parameters that are adjusted in order to ensure higher accuracy classification performance from the neural network.

[0112] With four directions θ and a single fixed distance d of one pixel, for example, a SGLDM

$$E = \sum_{i=0}^{N_g-1} \sum_{j=0}^{N_g-1} [s_\theta(i, j \mid d)]^2 \tag{8}$$

$$S = \sum_{i=0}^{N_g-1} \sum_{j=0}^{N_g-1} s_\theta(i, j \mid d) \log[s_\theta(i, j \mid d)]$$

$$H = \sum_{i=0}^{N_g-1} \sum_{j=0}^{N_g-1} \frac{1}{1+(i-j)^2} s_\theta(i, j \mid d)$$

$$R = \sum_{i=0}^{N_g-1} \sum_{j=0}^{N_g-1} (i-j)^2 s_\theta(i, j \mid d) \tag{9}$$

can be computed as a sum of co-occurrence matrices over the four directions in each ROI. Textural features can then be calculated from each SGLDM. For example, four different textural features that can be calculated from each SGLDM include energy (E), entropy (S), local homogeneity (H), and inertia (R). The inertia value is also referred to as "contrast". In this example, then, four SGLDM features for the set of angles θ can be calculated as follows for each ROI:

where $s_\theta(i,j|d)$ corresponds to the (i,j)-th element of the SGLDM for a distance d. The calculated values E, S, H, and R, for each of the image slices, can then be stored as the first 4N elements in the feature vector corresponding to the ROI for the currently selected class.

[0113] As an example, a 2×2 region **902** of an image is shown in FIG. **9**. The region includes 4 pixels, each of which can have an integral intensity level from 1 to 4 (i.e., N_g=4). The second-order joint conditional probability matrix $s_\theta(i,j|d,\theta)$ is therefore a 4×4 matrix **904**. In order to evaluate the numerical elements of matrix **904**, particular values of d and θ can be selected. For example, selecting θ=0 corresponds to evaluating probabilities along rows of region **902**. Selecting d=1 corresponds to evaluating probabilities for elements in region **902** that are separated by 1 unit (i.e., adjacent elements). With the selection of θ=0 and d=1 for region **902**, the values of the elements of probability matrix **904** are as shown in FIG. **9**.

[0114] In region **902**, pixel (1,1) has an intensity value of 1. Related to pixel (1,1), at a distance d=1 and angle θ=0, is pixel (1,2) with an intensity value of 3. Therefore, the probability value at position (3,1) in matrix **904** is 1. Pixel (2,1) in region **902** has an intensity value of 1. Related to pixel (2,1) at a distance d=1 and angle θ=0 is pixel (2,2) with an intensity value of 2. Therefore, the probability value at position (2,1) in matrix **904** is 1. In some embodiments, the next four calculations for each of the image slices in the ROI's feature vector can be derived from the magnitude of the complex 2D Fourier transform of the ROI. For example, the 2D Fourier transform can be calculated (e.g., using a 2D FFT algorithm, if the sub-sampling window width and length are multiples of 2) and the magnitude data stored in a matrix, wherein the DC frequency component is represented by the origin of the axis in the frequency domain. FIG. **5** is a schematic illustration of a sub-sampled ROI for which a 2D Fourier transform is calculated. The 2D Fourier transform data set can then be divided into four concentric regions **502**, **504**, **506**, and **508** based on frequency content. The outermost region **502**, for example, represents a portion of the sample image having the highest spatial frequency content.

[0115] The magnitudes of the spatial frequencies in each of regions **502**, **504**, **506**, and **508** can be integrated and normalized to the total signal magnitude. The integrated magnitudes form the next four elements in the ROI's feature vector, and each corresponds to a percentage of Fourier transform signal within a certain range of spatial frequencies.

[0116] In general, in embodiments, the spatial Fourier transform data can be partitioned into any number of selected frequency regions (subject to the spatial Nyquist limit) and the integrated intensities from these regions correspond to textural features of the image. Some or all of these textural features can be incorporated into the feature vector for the ROI.

[0117] The remaining two calculations in this present example of determining the feature vector can be derived from first order pixel statistics. For example, the ninth and tenth calculations can correspond to the mean and standard deviation of the pixel values within the ROI. In general, other statistical measures can also be useful as feature vector elements. These quantities can be derived from first order or higher order statistical measures (e.g., the variance in pixel values, which is derived from the second moment of the statistical distribution of pixel values).

[0118] Referring again to FIG. **3**, following calculation of each of the elements of the feature vector that corresponds to the currently selected class, the feature vector is stored. A logical decision **314** follows next. If feature vectors for all of the N_c classes have been calculated and stored, then subsequent neural network training steps, beginning with step **318**, are taken. Conversely, if feature vectors have not been calculated, then in step **316***a* class indicator i is incremented, which is equivalent to selecting a new class and its associated ROI, and the analysis for the newly selected class begins at step **308**.

[0119] When all of the feature vectors for the identified classes have been calculated, the next step in the sequence is step **318**, which includes selecting a sequence of the calcu-

lated feature vectors for use as training vectors corresponding to the N_c classes identified in step **302**. The set of training vectors can include multiple vectors corresponding to different ROIs for each of the classes. However, care is taken to ensure that each identified class contributes the same number of distinct training vectors to the training set in step **318**. This balancing of the relative abundance of different training vectors in the training set is important in order to ensure that the neural network is trained in unbiased fashion with respect to the different classes in sample images.

[0120] In step **320**, the set of training vectors is submitted to the neural network-based classifier for classification. The vectors are classified one-by-one in random order, and for each one the neural network develops an output estimate of what class the vector belongs to, and this is compared against the actual known class corresponding to the vector. The difference between the network output and the actual class is termed the error. The network is then adjusted using a method such as gradient descent back-propagation, or other error-adjustment techniques, which acts to adjust the network values and produce a reduced error value. When all of the training ROIs have been assigned by the network, the classification accuracy can be determined in step **322**, either manually by an operator or automatically by calculating a score that indicates, for example, what percentage of ROIs were classified correctly.

[0121] Logical step **324** includes a decision based on the accuracy of the classification of the training ROIs. If the accuracy is higher than a selected threshold (which, in some embodiments, can be set to 100% accuracy, for example) then the neural network is considered to have been suitably trained and the training sequence finishes in step **326**. However, if the accuracy falls below the selected threshold, then the steps involving classification of training ROIs are repeated. That is, training vectors are prepared as in step **318** and test classification of these ROIs by the neural network begins again. The vectors may be the same set used in the initial training, or may be a different set of vectors. Repeatedly training on a single set is productive as long as the error network adjustment continues to improve classification accuracy. In many embodiments, 100% accuracy is achieved on a first set of training ROIs. However, the threshold for successful training may be set lower than 100 percent if that is desirable. This may occur if one does not have perfect knowledge of class identity for the training ROIs, or if the samples themselves are highly variable and a wide range of training ROIs are employed.

Optimizing the Neural Network

[0122] Following successful training of the neural network-based classifier, the network can optionally be optimized with respect to the number of features used to classify sample images. Optimizing the network in this manner can increase the efficiency and speed of classification operations.

[0123] FIG. **6** is a flow chart **600** that includes an optional series of steps involved in optimizing a trained neural network. First step **602** includes generating a random sequence of training vectors to test the performance of the neural network. As before, the sequence of training vectors is constructed such that there exists an equal number of vectors corresponding to each of the N_c classes identified previously.

[0124] Step **604** includes choosing the number of neural network classification features N_f. Initially, the value of N_f typically consists of all the features that were calculated, for all image planes, which is the number of elements in the feature vector. Subsequent iterations of the optimization sequence can reduce the value of N_f according to the classification performance of the neural network.

[0125] In step **606**, the random sequence of vectors generated in step **602** is submitted to the neural network for classification. The classification of individual vectors is performed by the trained network in a manner consistent with the prior discussion. A feature vector is calculated for each ROI (e.g., based on one or more sub-sampled windows in the ROI), and the ROI is assigned to a particular class according to the known feature vectors for the various identified classes. In step **608**, a classification accuracy score is determined either by visual inspection (e.g., by an operator) or by calculating the fraction of correct classification results.

[0126] In order to assess the relative significance of each of the N_f features to the performance of the neural network, the mean feature value μ_j for each of the j classification features is calculated in step **610**. Calculation of a mean feature value can be accomplished, for example, by calculating a mean value of the elements in a feature vector corresponding to a particular class. The elements in the feature vector can be weighted equally or differently in performing the calculation of μ_j.

[0127] In a further step **612**, the weighted contribution W_j of each feature j of the N_f total features under consideration by the neural network is calculated according to

$$W_j = \sum_{k=1}^{N_f} \mu_j \gamma_k \tag{12}$$

where the γ_k values are the node-to-node coupling constants within the neural network. Using Equation (12), the weighted contributions of each of the features (which generally correspond to classes) can be evaluated. In step **614**, classification feature s having the smallest weighted contribution W_s is identified as the "weakest" classification feature and removed from the set of classification features considered by the neural network.

[0128] In steps **616** and **618**, a new random sequence of training vectors is generated according to the procedures discussed previously, and the training vectors are classified by the modified neural network, which now includes one less feature. A classification accuracy score is determined following classification of the vectors.

[0129] In logic step **620**, the classification accuracy score is compared against a selected accuracy threshold. If the accuracy score is higher than the threshold, then the removed feature is deemed to be insignificant enough that it can be permanently removed from consideration by the neural network. The number of neural network classification features N_f is reduced by one in step **622** and logical flow returns to step **610**, where new mean feature values are calculated for the newly reduced set of classification features in the neural network. In some embodiments, before logical flow returns to step **610**, the neural network can be retrained in order to adapt to the smaller number of features. This step is not necessary, but may be employed in some embodiments to improve the accuracy and/or speed of classification.

[0130] If the accuracy score is lower than the selected threshold, the removed feature s is deemed to have been significant after all, and is re-introduced into the neural net-

work in step **624**. This completes the optimization of the network in step **626**, and the network is then ready for use in classifying samples based on image sets.

[0131] If all features corresponding to a given input image plane are removed during the optimization process, that input plane is superfluous and need not be acquired in order to provide the classification signal. Further improvement in efficiency can be obtained by not acquiring such planes in future measurements, if the plane is not required for other purposes. The determination of which image planes are necessary can be made once, when devising a measurement protocol; or, it may be made and/or reviewed on an ongoing basis over time, in settings where factors such as sample variability may lead to changes in what image planes are necessary or helpful in making a classification.

Classification Using a Trained Neural Network

[0132] The procedure by which a sample is classified according to its image stack is shown in FIG. **7**. The figure includes a flow chart **700** that illustrates a series of steps in the classification procedure. In step **702**, a particular image stack for the sample is chosen for classification, and in step **704**, a number of regions N_r within the image stack are selected for analysis. In some embodiments, the regions selected are subsets of the entire image. In other embodiments, the entire image can be selected for analysis.

[0133] The image stack being selected for analysis may include one or more images. The images in the image stack may include one or more raw spectral images, one or more composite images, and/or one or more unmixed images. For example, in certain embodiments, the image stack may include one composite image to provide spectral and spatial information and one gray scale image to provide spatial information. In other embodiments, for example, the image stack may include a set of unmixed images. Furthermore, in some embodiments, for example, the classification may be applied to only a single image containing only spatial (and no spectral) information. In any case, the neural network is trained in anticipation of the type of image stack being selected.

[0134] In step **706**, a length l and width w of a sub-sampling window are selected. As discussed previously, the length and width of the sub-sampling window are typically chosen to be smaller than the mean length and width of each of the N_r regions selected for analysis. In addition, step **706** includes selection of window offset increments Δl and Δw. The offset increments are used to translate the sub-sampling window over the classification regions of the sample image in order to ensure that each of the pixels within the regions is classified at least once. In some embodiments, the values of Δl and Δw are both chosen to be smaller than l and w, respectively, so that at least some pixels are classified multiple times since each translation of the sub-sampling window to a new position leaves a fraction of the previous window's pixels within the new window.

[0135] In step **708**, one of the regions selected in step **704** is submitted to the trained (and optionally, optimized) neural network for classification. The classification of the pixels in the windowed region is performed in step **710**. The classification procedure is an iterative one, in which pixels within the selected region can be provisionally assigned a classification multiple times. The procedure begins by positioning sub-sampling window **802** within the selected region **800**, as shown in FIG. **8**. Sub-sampling window **802** has a length l in the x direction and a width w in they direction. The offset increments Δl and Δw are smaller than the length and width of the sub-sampling window, respectively.

[0136] In the first position of the sub-sampling window, each of the image pixels within the window is assigned a provisional classification based on the classification of the overall window region by the neural network using the methods discussed previously. The provisional pixel classifications can be stored within a pixel histogram for future reference. This corresponds to step **710**. Referring again to FIG. **7**, the next step is a logical decision **712** based on whether sub-sampling of the region is complete. If sub-sampling of the region is not complete, the sub-sampling window is then translated in the x and y directions by increments Δl and Δw, respectively, as shown in step **714**. The image pixels that fall within the new sub-sampling window position are then classified as before in step **710**.

[0137] The procedure is illustrated schematically in the lower part of FIG. **8**, in which window **802***a* represents the first position of the sub-sampling window and window **802***b* represents the second position of the window following translation. The classification of the pixels within the second window **802***b* by the neural network then proceeds as before. Note that the pixels that fall within shaded region **804** are classified a second time, since they are positioned within both windows **802***a* and **802***b*. The multiple classification of image pixels is a particular feature of certain embodiments of the methods disclosed herein.

[0138] Returning again to step **710** in FIG. **7**, the classifications of individual pixels are again stored in the pixel histogram, and then the sub-sampling window is again translated and the classification procedure begins anew for a new window position. This iterative procedure, consisting of steps **710** through **714**, can be specified to repeat for a selected number of window translations, such that a pixel classification histogram is built up.

[0139] Note that while FIG. **8** depicts the translation associated with step **714** as having both increments Δl and Δw, this is not necessary. For example, in some embodiments, the translation may scan horizontally, followed by a vertical translation when each horizontal scan across the selected region is complete, or vice versa. In certain embodiments, for example, each translation will correspond to a step of a single pixel and the translations across the region will generally result in each pixel being classified by number of pixels in the sampling window. Furthermore, in other embodiments, the translations need not be sequential. For example, the window translations can be systematic or random within the selected image region, although in some embodiments, an additional constraint that must be satisfied prior to termination of the classification procedure stipulates that all pixels within the selected region should be classified at least once, and preferably multiple times. Such a constraint is optional, however, and need not be imposed. Once the sub-sampling of the selected image region is complete, logical decision **716** determines a course of action based upon whether all of the selected regions of the sample image stack have been provisionally classified (e.g., a histogram of provisional classifications has been developed for every pixel in the selected regions of the selected image stack). If there are remaining unclassified regions, then counter i is incremented in step **718** (equivalent to selecting one of the unclassified regions) and classification of the selected regions begins at step **708** of flow chart **700**.

[0140] Alternatively, if each of the regions of the sample image have been provisionally classified, then the initial classification procedure is finished and control passes to step **722**, in which a final pixel classification step is performed based on the accumulated histogram data for each of the pixels. Due to the fact that pixels can be classified multiple times, entries in the classification histogram for particular pixels may not all be the same, and a pixel can be provisionally classified into more than one class.

[0141] A wide variety of algorithms can be used to establish a classification for a particular pixel from the histogram data. For example, the final classification of a given pixel can be the class to which the pixel was most frequently assigned. Alternatively, a more complex analysis of the statistical information in the histogram can be used to assign the final classification. For example, a pixel's classification can be established as the mean, median, or mode of the distribution of classifications for that pixel. Alternatively, more advanced statistical methods such as fuzzy logic or Bayesian logic can be applied to the histogram data to determine classifications for each of the image pixels.

[0142] In some embodiments, the histogram data can be used to "flag" particular regions of the sample according to classification. For example, if the histogram data for a particular pixel includes even a single instance in which the pixel was classified as belonging to a particular class, steps can be taken to ensure that the pixel is positively identified. Warning messages or sounds can be produced, or a sample image having the identified pixels highlighted for easy identification can be displayed. Flagging techniques can be particularly useful when tissue samples are examined for the presence of harmful agents or structures such as pathogens and cancer cells.

[0143] The final step **724** includes generating a classification map for the sample based on the final classification of step **722**, or more generally, on the provisional pixel classification histogram data generated in the earlier steps. The classification map can include, for example, an image of the sample with classified regions highlighted in order to enhance contrast. The map can include, in some embodiments, multiple images of the sample, where each image only those portions of the sample that belong to a particular class, as identified by the neural network. The classification map can also include numerical data specifying classified sample regions, and statistical information such as the distribution and relative abundance of various classes within the sample. This information is particularly useful when the classified regions correspond to different structural, chemical, or biological entities within the sample. The classification image map can be displayed on display device **118**, for example, and can be stored in electronic form on a storage medium by electronic control system **114**. Generation of the classification map completes the classification procedure, and generally yields accurate class data for a wide variety of samples.

Optical System Components

[0144] System **100** can include a wide variety of optical elements and devices for capturing images of a sample that are used in subsequent classification algorithms. Light source **102** can be an incoherent light source such as an incandescent lamp, a fluorescent lamp, or a diode. Light source **102** can also be a coherent source such as a laser source, and the coherent source can provide continuous wave (CW) or pulsed light. Light source **102** may contain multiple light source elements for producing light having a range of wavelengths (e.g., multiple diodes). When the light produced by light source **102** is pulsed (i.e., time-gated), various properties of the light pulses can be manipulated according to control signals provided to light source **102** from electronic control system **114** via communication line **132**. Light source **102** can also include various optical elements such as lenses, mirrors, waveplates, and nonlinear crystals, all of which can be used to produce light having selected characteristics. In general, light source **102** includes optical elements and devices configured to provide light having desired spectral, spatial, and, in some embodiments, temporal properties.

[0145] Light conditioning optics **104** and light collecting optics **110** can include a variety of optical elements for manipulating the properties of light incident on, and emitted from, a sample of interest. For example, light conditioning optics **104** and light collecting optics **110** can each include spectral filter elements for selecting particular wavelength bands from incident and emitted light. The spectral filter elements can include, for example, interference filters mounted on a filter. In some embodiments, adjustable filter elements based on liquid crystal masks can be used to change the spectral properties of the incident or emitted light. Liquid crystal based devices can be controlled by electronic control system **114** via communication lines **134** and **138**.

[0146] Light conditioning optics **104** and light collecting optics **110** can also include elements such as spatial light masks, spatial light modulators, and optical pulse shapers in order to manipulate the spatial distribution of light incident on, or emitted from, a sample. Spatial light modulators and other adaptive devices can also be controlled via communication lines **134** and **138** by electronic control system **114**.

[0147] Finally, light conditioning optics **104** and light collecting optics **110** can include other common optical elements such as mirrors, lenses, beamsplitters, waveplates, and the like, configured in order to impart selected characteristics to the incident or emitted light.

[0148] In general, detector **112** includes one or more measurement devices configured to detect and capture light emitted by a sample as multiple images of the sample. Detector **112** can include devices such as CCD arrays and photomultiplier tubes, along with their respective control systems, for acquiring the images. The adaptive optical devices in detector **112** can, in general, be controlled by electronic control system **114** via communication line **130**.

Software

[0149] The steps described above in connection with various methods for collecting, processing, analyzing, interpreting, and displaying information from samples can be implemented in computer programs using standard programming techniques. Such programs are designed to execute on programmable computers or specifically designed integrated circuits, each comprising an electronic processor, a data storage system (including memory and/or storage elements), at least one input device, and least one output device, such as a display or printer. The program code is applied to input data (e.g., images from the detector) to perform the functions described herein and generate output information (e.g., images showing classified regions of samples, statistical information about sample components, etc.), which is applied to one or more output devices. Each such computer program can be implemented in a high-level procedural or object-oriented programming language, or an assembly or machine

language. Furthermore, the language can be a compiled or interpreted language. Each such computer program can be stored on a computer readable storage medium (e.g., CD ROM or magnetic diskette) that when read by a computer can cause the processor in the computer to perform the analysis and control functions described herein.

Examples

[0150] The following examples are intended to be exemplary of the systems and methods disclosed herein, but should not in any way be construed as limiting the scope of the subsequent claims.

[0151] FIG. **10**A shows an example of a sample of rat blood that is classified according to some of the methods of the present disclosure. The blood sample includes 4 classes: background **1002**, red cells **1004**, monocytes **1006**, and polymorphonuclear neutrophils (PMNs) **1008**. A set of spectral images corresponding to incident light transmitted through the sample were collected and then transformed from measured intensities to optical densities (ODs). The resulting transformed images formed a spectral cube of image data.

[0152] The spectral image cube was unmixed into separate images corresponding to a red component **1010** and a blue component **1012** of the blood sample, as shown in FIG. **10**B. FIG. **10**C and FIG. **10**D show the results of this spectral unmixing operation. FIG. **10**C shows an example of an unmixed image corresponding to the red component **1010** and FIG. **10**D shows an example of an unmixed image corresponding to the blue component **1012**.

[0153] Following the unmixing step, a composite plane was generated by a linear ramp function used as the weighting function, so that the unmixed planes and composite plane formed a 3-plane stack. Next, training regions were selected on the image stack, and a neural network-based classifier was trained according to the selected regions. FIG. **10**E shows selected training regions superimposed on an image of the sample. Training of the neural network includes calculation of features related to the identified training regions. An expanded view of this process for the training regions is shown in FIG. **10**F. The left side of FIG. **10**F shows a view of an expanded region of a sample image that includes selected training regions. On the right side of FIG. **10**F, the selected training regions have been sub-sampled, and the sub-sampling windows are superimposed over the regions.

[0154] The trained neural network-based classifier was then used to classify the remaining regions of the images. The results are shown in FIG. **10**G. The image features corresponding to the background class **1002**, red cell class **1004**, monocyte class **1006**, and PMN class **1008** are all accurately determined and identified using the neural network-based classifier.

[0155] In another example, a 3-plane RGB image was generated from the same image cube of spectral images, and selected regions of the RGB image were used to train and optimize a neural network. This RGB image was generated by summing all the spectral bands in the blue to form a blue plane, summing all the spectral bands in the green to form a green plane, and summing all the spectral bands in the red to form a red plane. The result mimics what would have resulted if the scene were imaged using a conventional RGB camera. The trained and optimized neural network was then used to classify the remainder of the composite image. FIG. **10**H shows the RGB image, and FIG. **10**I shows the results of the classification operations carried out. The image features corresponding to the background class **1002**, red cell class **1004**, monocyte class **1006**, and PMN class **1008** are all accurately determined and identified using the neural network-based classifier.

[0156] The automated methods disclosed herein provide an effective means for classifying the blood sample.

[0157] A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

What is claimed is:

1. A method for classifying different parts of a sample into respective classes based on an image stack comprising a set of more than three spectral images, the method comprising using one or more processors to perform the following steps: classifying different regions of the image stack into respective classes based on the set of more than three spectral images, wherein each region comprises multiple pixels so that each classification involves both spectral and spatial information.

2. Apparatus comprising:

a means for obtaining an image stack comprising a set of more than three spectral images; and

an electronic processor coupled to the means and configured to classify different regions of the image stack into respective classes based on the set of more than three spectral images, wherein each region comprises multiple pixels so that each classification involves both spectral and spatial information.

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