Patent

Application of

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TITLE: AUTOMATED BONE MARROW RETICULIN FIBROSIS DETERMINATION- "AUTORETIC" METHOD

CLAIM OF PRIORITY

[0001] None

FEDERALLY SPONSORED RESEARCH

[0002] Not applicable.

REFERENCE TO A COMPUTER FLOW CHART

[0003] Program flow chart in the drawings

PARTIES TO A JOINT RESEARCH AGREEMENT

[0004] Not applicable

TECHNICAL FIELD

[0005] The invention relates generally to a system for automated light microscopic image analysis, specifically to computerized methods of converting reticulin stained brightfield color image of a bone marrow to an objective, clinically useful, statistical ordinal numerical data corresponding to the degree or grade of bone marrow fibrosis.

BACKGROUND OF INVENTION--INTRODUCTION

[0006] Quantifying crucial features of bone marrow biopsy includes basic parameters such as cellularity and fiber content as assessed by reticulin staining. These are important parameters to assess the dynamics of disease processes with importance on risk staging, survival and, therapy-related changes. Knowledge of the normal or abnormal range of reticulin is essential when the the grade or level is used as a factor in evaluating the hematologic disorders and metastatic cancer and these features are normally assessed by pathologists using the optical microscope.

[0007] Myelofibrosis is a concomitant cytokine mediated process of the bone marrow stroma. It has been associated with many different types of reactive conditions including, among others, autoimmune and granulomatous diseases, and a variety of neoplastic disorders. The latter include myelodysplastic syndromes, acute leukemic conditions and chronic myeloproliferative diseases (CMPD). In chronic myelogenous leukemia (CML) myelofibrosis was shown to be a significant predictor of therapeutic efficacy and outcome including engraftment after transplantation. In this context, therapy related effects on the stabilization or regression of fibrosis in chronic idiopathic myelofibrosis (CIMF) or myelofibrosis with myeloid metaplasia have gained increasing attention, particularly when considering novel therapeutic strategies.

[0008] Chronic myeloproliferative disorders or neoplasms (CMPDs) are a group of hematopoietic disorders that are clonal in origin but the stromal cells that produce reticulin fibers are non-clonal. These CMPDs include polycythemia vera, essential thrombocythemia, chronic idiopathic ***myelofibrosis*** (CIM, or primary myelofibrosis) and chronic myelogenous leukemia. These disorders have the potential to change from one to the other at any time. Chronic idiopathic ***myelofibrosis*** (CIM), in which stromal cells seem to play a profound pathogenetic role, is characterized by fibrosis of the marrow cavity, extramedullary hematopoiesis, splenomegaly, and anemia and leukoerythroblastic features in the peripheral blood. While myeloproliferation is known to be a clonal process, the accompanying stromal cell proliferation and fibrosis are believed to be a polyclonal reactive process that is likely to be due to increased intramedullary activity of a number of cytokines including TGF .beta., PDGF, FGF, EGF and calmodulin, among others.

[0009] The stromal cells provide a haven to various types of pre-leukemia and leukemia cells, non-Hodgkin's lymphomas (NHLs) and metastatic cancers (METs). Pre-leukemic clonal neoplastic conditions include myelodysplastic syndromes (MDSs) and myeloproliferative disorders (MPDs). Stromal cells are known to produce and/or respond to growth factors such as epidermal growth factors (EGF), Platelet derived growth factors beta and alpha, (PDGF-A, PDGF-B), fibroblast growth factor (FGF), vascular endothelial

growth factor (VEGF), and cytokines such as interleukin -1 (IL-I) or tumor necrosis factor apha (TNF a), partially explaining the interactive relationship between stromal cells and cancer cells. Stromal cells produce reticulin fibers which is measured as reticulin fibrosis or grade of fibrosis.

[00010] Bone marrow stromal cells represent a unique pluripotent or pluri-differentiated mesenchymal cell type, thus exhibiting preserved developmental "plasticity" but in many of the disorders listed above, the stromal cells differentiates to produce a bone marrow supporting framework that are dynamically altered and disordered towards stromal fibrosis manifested as reticulin fibrosis.

[00011] The optical microscope in the diagnostic and biomedical laboratory is routinely used by pathologists and research scientist to make diagnosis and perform experiments. These users perform these functions by visualizing cells and tissue sections that have been previously prepared and chemically stained in the histology or histochemistry laboratory. Every patient with a tumor suspected of cancer undergoes evaluation with the most critical pathway involving a tissue biopsy. The biopsy tissue is routinely fixed in formalin, processed in a tissue processor, embedded in formalin and serially cut in a microtome to give thin sections representing the diagnostic material. The diagnostic material then is a representative tissue section with tissue representations of both the cells and background supporting tissue and chemically marked with mordant dyes and indicia markers so as to visualized under a microscope. One of the routinely used dyes is the cytochemical stain reticulin and one of the common counterstain for cells is fast red cytochemical stain. The most common application of these two partner dyes is to visualize the stromal fibers of the bone marrow stromal support cells; determine if these are normal or increased. An increased reticulin fibers as visualized using reticulin stain is associated with a many bone marrow disorders or cancers.

[00012] The diagnosis is performed by examining the tissue optically using the objective lenses of the microscope in low and high power magnifications. The routinely stained hematoxylin and eosin tissue is examined first to visualize the cellular and tissue support appearance. The reticulin stain is then visualized to assess the degree of reticulin fibers and include the assessment in the final bone marrow report which is for the diagnosis of the disease. The current state of the art of diagnosis is to visually estimate the grade or the increase of the reticulin fibers quantity and based on this subjective interpretation render a diagnosis.

[00013] The diagnosis is either normal or increased. When it is normal according to a 4 point grading system, the assigned grade is less than grade 2. Abnormal mild increased fibers are more than 2 but less than 3, and moderate increase is greater than or equal to 3 but less than 4 and marked or severely increased reticulin fibers is greater or equal to grade 4. In general, 0 to 2 is considered normal and 3 to 4 is considered increased. This grading system is widely adapted according to a scoring system previously published (Bauermeister DE. Quantification of bone marrow a-a normal range. American Journal of Clinical Pathology 1971:56:24-31. and Adapted from BJ, Bain, July 2001, Bone Marrow Pathology Textbook.)

[00014] No tool is currently available to use computerized image analysis to count and display these reticulin fibers to help the pathologist or hematologist to diagnosis the

grade of the reticulin fibers in the bone marrow. Measuring the amount and identifying these fibers are crucial in making decisions for diagnosis or prognosis, yet the diagnostic practice relies on a subjective approach, even though patient outcomes and treatment decisions are at stake. The latter practice is the standard of practice, not because it is the optimal way, but because of an absence of an automated reticulin-quantifying instrument attached to the microscope. This manual practice is subjective, error prone, and often gives wide range of results that depends on the level of microscopist's skill. This is due to difficulty in counting fibers accurately and in a timely manner. To analyze reticulin fibers, we only have two techniques: manual estimation and manual morphometry.

[00015] Manual estimation is being done by pathologists. The method is using the microscopic appearance as visualized using the microscope binocular objectives viewed by a pathologist or a scientist. The user then view the images under low power or high power magnification objectives and estimate the degree and amount of reticulin fibers and mentally correlate these parameters to previously viewed bone marrows and descriptive criteria as outlined below. Manual estimation is quick but very subjective because the pathologist guesses the amount of the fibers by visualizing the bone marrow biopsy magnified about 200x and moving the field of view from one end of the biopsy to the other end.

[00016] In contrast, research approaches uses morphometry by employing scanners and/or digital cameras equipped microscope to capture images of reticulin stained marrow and manually count the density of fibers vis a vis a displayed or outlined grids overlayed on a digital frame area or areas. Morphometric examination is done by quantifying the density of fibers as assessed in relation to the cellularity of hematopoietic tissue. This feature is especially important in order to avoid a false impression of a reduced fiber content in fatty and/or edematous bone marrow samples after treatment. Often the technic relies on either a digitizing display tablet to score the reticulin fibers or a digital image frame with superimposed grids representing the dimensions of the image frame to count the reticulin fibers per grid unit area to arrive at the total or average reticulin per digitized frame reference. This method is tedious and not routinely applicable to a diagnostic work flow in a clinical laboratory where a diagnosis is made in less than 30 minutes using visual inspection. A morphometric examination may take a long time, sometimes measured in hours. This is due to manually counting these fibers per grid area where total microscopic grids could be in the tens or hundreds per bone marrow biopsy.

[00017] The criteria for grading have changed over the years and several approaches have been used. The currently most popular system is based on the 0 to 4 grade system based on Bauermeister or the 0 to 3 point system based on Thiele. Both methods are recommended as best scoring based on the chapter on Primary Myelofibrosis published in the WHO 2008 handbook of hematologic malignancies (Swerdlow et al, 2008). The difference between the two technics is the type of staining used; the former uses only the reticulin fibers and the latter uses a combined reticulin and trichrome staining for thicker collagen. Conversion between the technics is straightforward using the published criteria. Most of the centers use reticulin and a few add trichrome when needed as most of the fibrosis is made up of reticulin fibers and severe fibrosis even with thicker collagen is also visualized using just the reticulin stain.

[00018] Table 1. Survey of previous semiquantitative scoring systems on myelofibrosis in normal bone marrow and chronic myeloproliferative disorders

Authors	Number of grades	
Bauermeister	6	1971
Manoharan	5	1979
Lazzarino et al.	4	1986
Dekmezian et al.	4	1986
Beckman and Brown	6	1990
Buhr et al.	4	1993, 2003
Georgii et al.	4	1996, 1998
Thiele et al.	4	1996, 2003
Kvasnicka et al.	4	1997

[00019] The Criteria for Reticulin Grading as used by the current inventive system is based on the following descriptors using the reticulin stain only.

0 No reticulin fibers(normal)

1 Occasional fine individual fibers and foci of a fine fiber network(normal)

2 Fine fiber network throughout most of the sections, no coarse fibers(normal)

3 Diffuse fiber network with scattered thick coarse fibers, with crossings, occasional megakaryocytes encircled (Reticulin Fibrosis)

4 Diffuse often coarse fiber networks with crossings, with back to back fibers and many cells including megakaryocytes encircled (Myelofibrosis)

[00020] In this scheme, '0-2' fibrosis is conventionally interpreted as normal and '3-4' fibrosis is increased reticulin. This convention is based on the study by Beckman et al. (Arch Pathol Lab Med. 1990 Dec;114(12):1241-3) who reported on grading reticulin stains of bone biopsy specimens from 100 hematologically normal patients documented that the normal amount of reticulin in the marrow is low. Twenty-seven percent of the patients had marrow reticulin grade 0 using the Bauermeister scale, 42% had grade 0-1, 27% had grade 1, and 4% had grade 2; no patient had a Bauermeister grade 3 or 4 reticulin level. Therefore, scores less than 3 are normal.

[00021] We have applied this grading system and used a novel computerized algorithm on real patient sample materials. Details are as follows:

[00022] Here, we describe a novel AUTORETIC software results in comparison with the pathologist manual grading. A test and control group of images are compared. Test group has 65 patients, 23-80 years of age, M:F ratio 1:1.6 of patients with various hematological diseases. The biopsy reticulin stains were performed on 40 Myelodysplasia/AML/Myeloproliferative neoplasms including chronic myelogenous leukemia, polycythemia vera, myelofibrosis, 7 myeloma, 17 lymphomas, and 1 systemic mastocytosis. The reticulin staining was performed using the Ventana Nexes automated reticulin system (Ventana, Tuczon AZ) as part of the standard technic.

[00023] Control group of similar diseases comprise 248 cases, with marrow biopsy stained with a non-automated reticulin technic, using manual cytochemistry and using a different microscope CCD setup, is graded by two different pathologist, but with images

likewise run in AUTORETIC to compare reproducibility staining was performed using VENTANA Nexus automated system. A 20x digital color image was captured: average of 3 frames (1-7) per case. The results of manual grading by two pathologists are compared with the automated grading(averaged) by AUTORETIC software that was developed and provided by IHCflow/GreenGreat corp. using proprietary algorithm. Diseases include. Grading was performed based on Bain's criteria as modified from Bauermeister. (BJ Bain etal, 3rd Ed, Bone Marrow Pathology, 2001).

[00024] The AUTORETIC analysis finished 2-3 seconds with continuous grading rounded to 0+, 1+ 2+ 3+, 4+ output. Agreement between the pathologist and computer was judged true if the difference between grades is 0.5 or less.

[00025] Results demonstrate positive strong correlation between the manual grading and computer grading using our test and control groups. In the test group, the mean reticulin manual grading is 1.8 (95% CI 1.58 - 2.07) vs AUTORETIC mean of 1.734 with 95% CI(1.47 - 1.99) with no significant difference between the mean& SD. The correlation was high with r 0.8699 (non-parametric Spearman $p<0.0001$).
In the control group, the correlation was likewise high with r 0.7687(Spearman, 95% CI 0.71-0.82). In conclusion, the mean and SD for manual and AUTORETIC program are similar and they highly correlate with each other, indicating the software is a rapid, accurate and reproducible computerized technic that will be objective and useful in clinical bone marrow analysis applied to reticulin stain using automated stainer or manual technic. In addition, the program is robust and adaptable to variability of conditions and is applicable to bone marrow stained manually or automatically, digitized in a different microscope, or scored by a different users.

[00026] The current state of the clinical art in pathology diagnosis allows the pathologists to either make a judgment call for a positive or negative result of reticulin stain or semiquantitatively grade the fiber content and give an estimate based on the pathologist subjective feel of the extent of fibers deposition. Routinely in pathology practice, a biopsy of the bone marrow is stained with hematoxylin and eosin, Periodic Acid Schiff (PAS), Prussian Blue for Iron deposits, and Reticulin stain are applied on the slide-based tissue sections to create a panel of slides to rule in or out a diagnosis based on morphologic appearance of the tissue. Most of diagnostic pathology, whether a small office or a large reference laboratory, uses these routine stains as part of a standard of practice. General references such as Thompson (Selected Histochemical and Histopathological Methods, C. C. Thomas, Springfield, Ill., 1966), Sheehan and Hrapchak (Theory and Practice of Histotechnology, C. V. Mosby, St. Louis, Mo., 1973), and Bancroft and Stevens (Theory and Practice of Histological Techniques, Churchill Livingstone, New York, N.Y., 1982) provide the technical methodologies.

[00027] Current image analysis in diagnostic centers uses specialized tools to semiquantitate protein receptors such as hormone receptors, lineage markers, proliferative markers and others. Yet none of these diagnostic centers have an automated method with which to quantitate reticulin stained slides. Automated detection of stromal structures such as reticulin fibers in tissue has lagged behind quantitation of antigen in tissue and cells for prognosis and diagnosis.

BACKGROUND--PRIOR ART

[00028] US Patent Application Publication 2009/0048785 A1 published on Feb 9 2009 to Katzir et al titled Methods And Systems For Analyzing Biological Samples performs digital microscopic analysis of tissues including the bone marrow. This invention covers attempts to examine and scan for nucleic acid, proteins, subcellular moieties and apply computerized digital data analysis. The invention does not examine bone marrow fibrosis, reticulin stains, collagen, stromal elements and hence does not have the methodologies germaine to the topic of the present invention.

[00029] US Patent Application Publication 2009/0040157 A1 published on Feb 12 2009 to Meier et al titled Raman Difference Spectra Based Disease Classification performs digital microscopic analysis of tissues including the bone marrow. This invention covers attempts to examine and scan for nucleic acid, proteins, subcellular moieties and apply computerized digital data analysis. The invention uses raman spectral analysis and not brightfield method and does not examine for or analyse bone marrow fibrosis, reticulin stains, collagen, stromal elements and hence does not have the methodologies germaine to the topic of the present invention.

[00030] US Patent 7359548 published on April 15 2008 titled Method And Apparatus For Automated Image Analysis Of Biological Specimens uses digital microscopy analysis of tissue including the bone marrow tissue using ratio of brightfield color pixels to detect cells of interest but does not detect marrow stroma, reticulin, fibrosis or collagen fibrosis. The methodologies as described are applicable only to the identification of cellular specimen in tissue and hence do not have the methodologies germaine to the topic of the present invention.

[00031] US Patent published 7359536 published on April 15 2008 by Hays et al entitled Automated Method For Image Analysis Of Residual Protein is an invention that relates generally to light microscopy and, more particularly, to automated analysis of cellular specimens containing stained markers for cytochemistry of cells for alkaline phosphatase and esterase butyrate. These are intracellular enzyme markers of cell lineage and not related to extracellular matrix products such as reticulin or collagen fibers as is the subject and method related to the current invention.

[00032] US Patent published 7292718 published on November 6 2007 by Douglass et al entitled Color Space Transformations For Use In Identifying Objects Of Interest In Biological Specimens relates to analysis of cells and cellular objects and not extracellular products using pixel transformation of colored pixels. The methodologies are pertinent to the cellular objects and not applicable to extracellular matrix products such as reticulin, collagen, stromal elements as is related and pertaining to the current invention.

None of the previously patented or application subject matters deal with bone marrow reticulin fiber examination and analysis using automated digital means as in related and germaine to the current invention.

Additional Commercial Products:

[00033] PAXIT is a commercial turnkey product that belongs to a large group of turnkey image analysis products for image analysis that uses generic digital morphometry for medical application and similar to that large group of products, does not have a customized module for reticulin staining or bone marrow cellularity or stromal analysis thereof.

OBJECTS AND ADVANTAGES

[00034] It has been found, however, that present prior art apparatus and methods fail to address the reticulin fibers analysis and meet the demand for a low cost, efficient, customizable imaging microscope that is capable of extracting the reticulin fibers over color image obtained by brightfield light.

[00035] A problem found in manual and alternative digital systems is that they are capable of imaging a set of pixels representing the stained object over all the other digital objects without consideration of the bone marrow cellularity. Bone marrow cellularity changes with age, disease, treatment and varies over time in response to myelo-ablative and - treatments. By incorporating the bone marrow cellularity information, the reticulin stain per marrow unit area is more accurately quantitated in an automated manner.
A related module as an alternate embodiment also part of the family of patent application by the inventors is herein referenced.

[00036] Another problem with available systems is the need for special filters, and use of non-routine stains like fluorescence based markers or tedious manual micrometers attached to the microscope increasing the complexity in the system optics as well as introducing subjective, tedious, time prohibitive analysis along with selective bias. The present invention uses routinely available pathologist microscopes with CCD color camera and computer modules.

[00037] Accordingly, besides the objects and advantages of the "AUTORETIC" method on a slide in our above patent, several key objects and advantages of the present invention include:

[00038] a) Use of routinely stained slide, either manually or automatically stained by automated staining machines, that are readily available in the diagnostic laboratory for pathologist and scientists.

[00039] b) Use of routine diagnostic microscope with outfitted CCD RGB camera available in most pathologists or scientists office.

[00040] c) Use of combine bone marrow cellularity image analysis result along with reticulin fiber result per marrow unit area instead of the pixel and area-based image analysis.

[00041] d) Use of a graphic display of the actual image overlayed with the reticulin fibers digital image allowing verification of the accuracy by the user. Optimal evaluation of these results are then rapidly visible and accessible for approval by pathologists or system modification based on the actual visualized original tissue and digitized representation of reticulin distribution over the actual colored image.

[00042] e) Use of a novel thresholding algorithm that dynamically adjust for staining and sectioning variabilities.

[00043] f) Use of a novel thresholding algorithm that dynamically adjust for a wide range of light intensity of the transmitted light microscope.

[00044] g) Use of a combination of the above to provide a new and improved apparatus and method that converts colored digital image of reticulin stained marrow tissue into a verifiable display, with grading results along with other marrow cellularity parameters.

[00045] Slide based Reticulin cytometry as being described herein will aid in slide based diagnosis in providing an automated functionality to histochemically stained cells fixed on a microscopic slide.

[00046] Our approach is advantageous than prior art because there is no need to use an expensive imaging spectrometer or spectral microscopes as one may approach the variable color segmentation problem. Moreover, there is also no need to identify pure color and measure color differences as colors are often mixed in tissue. There is also no need to have a special light such as UV (ultraviolet) , multispectral or confocal optics. An ordinary microscope with camera using brightfield microscopy is all that is needed for obtaining the images.

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DEFINITIONS OF TERMS

[00080] Reticular fiber or reticulin fiber is one or more types of very thin and delicately woven strands of type III collagen (stromal protein), and these strands build a highly ordered extracellular network and provide a supporting network for tissue including the bone marrow. Many of these types of collagen have been combined with carbohydrate. Thus, they react with **silver stains** and with **periodic acid-Schiff** reagent

but are not demonstrated with ordinary histological stains such as those using **hematoxylin**.

[00081] A preferred embodiment of the cell indicia marker and dye is further described as follows. The marker and dye staining techniques may be broadly classified in at least three categories:

[00082] 1) Stromal fibers staining, which may be based on chemical attraction or reaction to metal salts such as silver nitrate; in our method this may be a silver stain. Silver nitrate is the metal salt that forms insoluble silver phosphate with phosphate ions which are present in the tissue such as collagen or reticulin fibers. When subjected to a reducing agent, usually hydroquinone, it forms black elementary silver that precipitates on the reticulin fibers outlining them as black or brown black in color.

[00083] 2) DNA chemical conjugation, such as Feulgen staining, and characterized by covalent binding, with acid hydrolysis of DNA; Examples include Thionin for the Feulgen staining technique for nuclear DNA and DAPI for nuclear reaction.

[00084] 3) Nuclear and cell counterstain using cytochemical electrostatic interaction indicated by dye-cell chromatin reaction. Examples: Fast or Neutral Red as used in this method which react to both the nuclei and the cytoplasms of cells in the bone marrow, and others include ethyl green stain, hematoxylin, methyl blue or eosin. The sources of staining affinity noted above are referred to as stain-cell attractive forces. A more expansive discussion of staining and staining mechanisms may be found in "Standardization and Quantitation of Diagnostic Staining in Cytology," edited by M. E. Boon and L. P. Kok.

[00085] In the above-noted methods, the apparatus for the present method provides a dual thresholding method to distinguish the areas stained by the fast red [cytoplasm and nuclei] and the areas stained by the reticulin silver stain[reticulin fibers].

[00086] Bone marrow is the organ in the body that produces blood. They are normally located in long and flat bones such as the pelvis, sternum, bones of the skull, legs and arms.

[00087] Biopsy is a procedure that use a sharp pointed needle with a hollow core to extract bone marrow or other tissue from the human body.

[00088] Silver staining is the use of silver to stain histologic sections. This kind of staining is important especially to show proteins (for example type III collagen). It is used to show both substances inside and outside cells. Silver nitrate forms insoluble silver phosphate with phosphate ions. When subjected to a reducing agent, usually hydroquinone, it forms black elementary silver. Silver staining is used in light microscopy. The metallic silver particles are deposited on sensitised reticulin fibres and are then easily seen in the microscopic preparations. Silver stain aids in the perception of reticular fibers.

[00089] The Gordon Sweet Reticulin method as is routinely known in the histology art used with modifications in both the manual and automated fibers is as follows. The tissue is treated with potassium permanganate to produce sensitized reticulin fibers to make them react to silver. The diamine silver solution using pH 9.0 precipitates on these sites because of reduction with formalin. Excess silver is removed by washing with Hypo solution. Counterstain with neutral red or fast red for two minutes. Reticulin fibers are black and nuclei of cells are red. The results are shown in figures at the end of this application.

[00090] A digital image will be defined for the purposes of describing the invention as a two-dimensional collection of points with intensity I (x,y) at coordinates (x,y). Color images are replaced with color RGB(x, y) at coordinates (x, y).

[00091] A histogram of a picture is a plot of intensity or color versus the frequency of occurrence. The range of intensity of a picture is often small compared to the range available on a system. The global real color image is the ground truth that is referenced by the user to collect histogram characteristics--which generally fall into bimodal or multimodal categories. The multimodal categories of global image lends itself a type of histogram thresholding mode usually by entropy parameter while the isodata parameter worked better with bimodal histograms.

[00092] Mathematical morphology is an approach to image processing which is based on the shape of the objects processed. Haralick et al. described in "Image Analysis Using Mathematical Morphology", but the equations have been reformulated based on Boolean arithmetic instead of set arithmetic to facilitate the conversion to computer programs. The following logical operations are used: OR, AND, EXOR for binary images. Dilation is an operation that spreads out the marked pixels and has the effect of reducing noise and filling small holes. Erosion is an operation that contracts the marked pixels and has the effect of thinning the object and expanding holes. The most common dilation and erosion operations have as input an image and a structuring element known as the dilation or erosion mask. The shape of the structuring element known as the dilation or erosion mask depends on the application. Dilation and erosion are often performed in pairs.

[00093] Objects Operations and Counting (OOC) usually refers to the techniques of locating marked objects and obtaining information about them. Assume that the pixels in the objects all have value 1 and the background pixels all have value 0. The technique for locating the objects is well known and uses region of interest and the corresponding identified objects represented by bitplanes, masks, or binary objects. The previously processed binary image is scanned until an object pixel (which is the starting pixel for boundary tracing) is encountered.

[00094] Hue singularity where the hue and saturation is undefined when RGB=1 or 0, i.e., the darkest and brightest spots, respectively. Many systems fail without removing singularities.

[00095] Gray-value morphological processing using iterative Isodata technique was developed by Ridler and Calvard and has appealing functionality in their relative

insensitivity to brightness or darkness range of the histogram, but is readily influenced by the histogram shape.

[00096] Isodata mode is an automated method. The histogram is initially segmented into two regions using a starting threshold value such as the half the maximum dynamic range. The sample mean associated with the background and foreground pixels are computed for the gray value. A new threshold value is computed as the average of these two sample means. The process is then repeated, until the threshold value does not change anymore. After the algorithm is applied, the population of interest is separated. In our example, we applied this principle to color images, and when the histogram is based on the degree of brown staining or lack thereof, the positive and negative cells are separated as two binary objects.

[00097] Gray-value morphologic processing using the entropy thresholding technique was developed by Johannsen G, Bille J. Entropy algorithm is an automated mode that dynamically adjust to the image histogram distribution but is likewise relatively insensitive to the brightness range. The method divides the histogram into two part, minimizing the interdependence between two parts, measured in terms of entropy. The grey level that performs this division will be the threshold value. As a condition, the user may specify the fraction of the image that minimally should be assigned to be a foreground object. The algorithm then searches for the minimal entropy within this constraint. In our example, we applied this principle to color images, and when the histogram is based on the degree of brown staining or lack thereof, the positive and negative cells are separated as two binary objects, with the added bonus of an adaptive parameter in the form of the fraction epsilon.

[00098] Bitplane sculpting: In both these isodata and entropy modes, the user specifies the part of the image to consider for the computation of the histogram. In our example, the parts of the image pre-processed by RGB is used, then the intersection of these images are used, then the resulting region of interest are transformed to different color value, and the thresholding is applied to these narrower tier of images. The result of the thresholding operation is stored in one of a number of bitplane images used in bitplane sculpting operations and the value is also stored and accessible.

SUMMARY OF THE INVENTION

[00099] The major problem overcomed by this invention was the manual estimation of reticulin stained reticulin fibers which until now is being estimated subjectively and manually.

[000100] The automation is done digitally using a novel algorithm that overcomes the staining variability and wide range of lighting; and identifying the silver stained dye and the background chromogens that identifies the cell as well as the cell nuclei using counter stain dyes such as neutral or fast red and hematoxylin; the latter for the purposes of extracting the bone marrow cellularity; and integrating these automated results in daily diagnostic pathology practice so the user could easily use the tool and visualize the results in minimal effort and time. The current invention provided solutions to the above objectives and is easily performed and implemented by a person ordinarily skilled in the art of digital image analysis.

BRIEF DESCRIPTION OF THE DRAWINGS

[000101] The patent or application file contains at least one drawings executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[000102] FIG. 1 shows the exemplary components needed to accomplish the processes of the invention.

[000103] FIG. 2 shows the exemplary microscope and CCD camera and digital image obtained from tissue on microscopic slide.

[000104] FIG. 3 shows the exemplary tissue on microscopic slide and the magnified microscopic image of cells depicted as raw reticulin images visualized under a microscope as tissue sections and the corresponding reticulin only digitized results excluding other objects that are not reticulin fibers.

[000105] FIG. 4a, b,c shows the exemplary true color image frame 512.times.474 pixel resolution of the microscopic section containing the objects of interest including the reticulin stained fibers with a background of counterstained bone marrow cells and the corresponding computer implemented results.

[000106] FIG. 200 depicts an example of a method **200** that can be employed following sampling of reticulin images by apparatus as shown in FIG. 1 ~ FIG. 3.

[000107] FIG. 100 depicts an example of a method **100** that can be employed following sampling of reticulin images by apparatus as shown in FIG. 1 ~ FIG. 3.

DETAILED DESCRIPTION OF THE INVENTION

[000108] FIG. 1 is a block diagram of the interface of the system. The system includes a human operator or an automated slide delivery system, to place and select the tissue to scan for low power color image. The image is scanned of 3 channel RGB monochromatic planes which are sent to the main program. The main program and its data storage are in preferably a pathology workstation with monitor display or alternatively located in a remote server.

[000109] The general purpose computer 1, preferably a personal computer (PC) FIG. 2 controls the operation of the image processor board preferably a Pentium with PCI bus advanced chip, running Windows 9X or greater or a PowerPC with PCI bus running OS 8.5 or greater and able to run executable programs. The frame memory of the image processor is memory mapped to the PC which performs most of the complicated computations that the image processor cannot handle. This includes searching and other operations requiring random access of the frame memory. With careful planning, the image processor can perform selected functions in parallel with the computer. The use of a PC also facilitates the transfer of data to other software packages that are readily available. The user interacts with the computer via a keyboard and a mouse. An industry standard interface circuit and ports with software to connect to the internet is preferred.

The output device is provided to allow the user to view the data and the results obtained. A graphics color monitor and printer capable of displaying the entire video frame is required.

[000110] FIG. 2 show a microscope 3, a color CCD camera 5, a general purpose computer 1 equipped with a special purpose image processing board, and an output device 1 such as graphics monitor or printer. Each of the different components will be discussed in greater detail below.

[000111] The trinocular microscope 3 is equipped with set of eyepiece objectives 2 for the human operator to visualize the image obtained from microscopic slide 8 on stage 9 and illuminated by a stable light source 10. The operator focuses the slide view wherein the white spectrum of visible transmitted light image 7 is on focal plane of the eyepieces field of view located above 2 and on the imaging plane of the camera 5. The microscope is equipped with a plurality of objective lenses 6 to perform a plurality of magnifications; with 20.times objective the preferred embodiment because of optimal level of cell detail obtainable with this objective magnification. The image from the CCD camera 5 is funneled in 3 channels representing the blue, green, and red monochromatic image planes, respectively.

[000112] The camera 5 is directly connected to a regular trinocular microscope 3 via an optical tube such as a trinocular head, and in alignment with the optical path of the transmitted image of the tissue on slide 8 thru the two objectives 2 which are also integral part of the trinocular head. The camera 5 is preferably a 3 channel CCD RGB camera with separate outputs into 3 channels corresponding to red, green, and blue monochrome images. Preferably, the sample image could be saved as 24 bit depth in RGB color. A CCD chip with a 3 channel 1600.times.1200 active pixels (1.2 million) CCD (Charge-Coupled Device) with 7.4 um square pixels KAI as sold by Diagnostic Instruments. The bit depth is sampled at 30 bit RGB and could be saved as 24 bit or more for enhanced sampling of details. The overall operation of CCD equipped cameras is well known in the art. The camera image could be saved as 512.times.474 pixels or any other predermined spatial format to be used in the analysis.

[000113] The output of the camera 5 is digitized and stored for reference and manipulations. This task is accomplished by an image processor board contained within the general purpose computer 1. Alternatively, the image processor capable of supporting 24 bit RGB of the desired monitor, can take the form of a separate unit coupled to the general purpose computer 1. An image processor preferably is a single board, real-time image processor designed to be interfaced with IBM PC-AT's and compatibles, via a preferred PCI bus card, although other image processing devices may be readily employed. The image processor could at least digitize 512.times.474 pixel images from the camera with 8-bit precision (256 gray levels) per channel. The video card software driver should be capable of saving images in a plurality of standard image file formats, including TIFF, JPEG, PICT and others.

[000114] The tissue and slide component is shown at FIG. 3. Current algorithms aim to extract a precise cell boundary for mensuration especially relevant in cytologic images. In FIG. 3, slide 11 contain on its surface a cut section of tissue 13 identified as belonging to a patient identified by ID 12. The projected microscopic color image 14,

visible in previously mentioned microscope objectives and monitor, displays the cell objects of interest along with the reticulin fibers. These objects are the converted to digitized reticulin fibers only displayed in 15.

[000115] In FIG. 4a, b,c , are tissue frames showing the reticulin fiber stains and the corresponding computer automated results of each grades displayed as 16 as raw reticulin stains as visualized in the microscope of the user and captured as digital images with exemplary grades from 1 to 4. Note the variability in background color which did not interfere with the automated analyis.

[000116] These digital images as raw images on the left panel with corresponding grade as manually estimated by pathologist user and the AUTORETIC grades displayed as real number and as rounded number show in figures 4b, 4c labeled as image 17 and image 18, respectively for example of different grades.

[000117] The invention uses multiple layers of processing and some extra explanations is detailed below. As image data passes through various stages, with each stage applying bitplane sculpting for thresholding providing finer and finer discrimination of objects from non objects. The method uses a novel multi-stage thresholding and segmentation algorithm based on multiple color channels in RGB and HS I spaces. The algorithm uses auto-thresholding on red and blue channels in RGB to get the raw working image of all cells, and then refines the working image with thresholding on hue and intensity channels in HS I, and further separates different classes of cells by auto-thresholding within the working image region.

[000118] FIG. 1 shows the initial steps of the invention with human input and human reference for the "ground truth" that does so in FIG. 2 by either looking under the objective lenses 2 or by referring to image in the display monitor in 1 after focusing the microscope and adjusting for the optimum light setting, this subject treated more below. In one embodiment of the invention, the computer system 1 processes a 20.times. Magnification field of view (FOV) to be displayed after processing in CCD camera 5.

[000119] As some control is necessary to avoid camera saturation, or inadequate exposure in any one of the color bands, balancing is performed automatically by utilizing a white light calibration by obtaining first an image of clear slide 11 in location without blemish or without tissue 13 and using the software for setting the white balance before human operator starts using this system.

[000120] The image scanning begins by providing for a plurality of scanned images from the microscope 3. A preferred embodiment includes a software module with calibrated brightness filter to get the optimum light exposure. The human operator or the automated delivery system has to also set the optimal transmitted lighting using a rheostat mechanism controllable to a predetermined range of brightness. In one embodiment, the brightness is set using an Olympus microscope equipped with a 30 watt halogen light source and a range of setting from 1 to 10, to a range between 5.5 to 6.5 on the dial. This setting is optimal even though image analysis on test systems was stable with a rheostat setting from 4.0 to 7.0. We also found that image analysis results are stable even without a blue 80A Tiffen filter, when the condenser is down, or when the light bulb is just replaced with a new one. In those extreme settings, the whole image goes from yellowish

saturation to bluish saturation from low to high number of rheostat respectively. Because of the robust automation that adjusts to the feature inherent in the image, the results are noted to be accurate despite the brightness variation in contrast to prior art results which are exquisitely sensitive to brightness variance. Preferably, our algorithm has, outside this predetermined range of light intensity, a trigger for an error signal or prompt for correct adjustment.

[000121] A typical imaging system may use an incandescent halogen light bulb as a light source. As the bulb ages, the relative amounts of red and blue output can change. The tendency as the bulb ages is for the blue to drop off more than the red and the green. To accommodate for this light source variation over time, white balancing process as described above. The brightness of an image is provided by histogram fluction determines how many pixels within a gray scale FOV have a certain image intensity and cut off values of too dark or too bright are predetermined to warn the user of suboptimal lighting of the images. This procedure is preferably using a wide range of 120 and 230 as brightness cutoff over a range of 256, of low and high end respectively. The optical density of the total pixel of the grabbed image are summed up and averaged to give the mean brightness. The total pixel number is divided by the mean brightness to give the total average frame brightness. This value is used as the cutoff.

[000122] In FIG. 4a, as one exemplary drawing, the whole color frame of the source image shows the raw bone marrow images of reticulin stains in grades 0 to 4.

[000123] The FINAL RESULTS of the computer system 1 running the algorithm are outputs based on the above set of is an overlaid image with the grade of reticulin in both the real value and the rounded value to the nearest grade is shown in figures 4 b and 4 c.

[000124] In Figure 4b, as one exemplary drawing, the images of bone marrow reticulin fibers are converted to numerical grades 0 to 2. The conversion uses a rounding system so that the real number results are converted to usual numerical rounding to the nearest integer number.

[000125] In Figure 4c, as one exemplary drawing, the images of bone marrow reticulin fibers are converted to numerical grades 3 to 4. Note the red colored cells and nuclei are removed from analysis for a more accurate grade. Grading is done using a set of criteria as outlined in the description of the algorithms as outlined below.

[000126] By applying image processing and analysis algorithms method **200** can display fibers in reticulin images as well as corresponding grading results. Method **200** can be implemented as either computer hardware or software or a combination of both, and can be implemented as a stand-alone program or a program combined with image sampling and preprocessing programs. Method **200** contains automated image segmentation and morphological processing, numerical image data analyzing, and user interfacing.

[000127] At **201**, an image subject as being analyzed in **200** is loaded into computer memory separated into three distinct channels labeled as Red, Blue and Green channels.

[000128] At **202**, automated histogram thresholding, such as ISODATA or Entropy thresholding, is applied to the Red channel pixels to segment foreground objects from the background and these foreground pixels are assigned to a mask labeled mask A.

[000129] At **203**, automated histogram thresholding, such as ISODATA or Entropy thresholding, is applied to the Green channel pixels to segment foreground objects from the background and these foreground pixels are assigned to a mask labeled mask B.

[000130] At **204**, a logical <OR> operation is applied to combine results of prior two steps, mask A and B, to get primary region of interest, as mask C.

[000131] At **205**, first the Red channel is masked with mask C and all pixels out of mask C are removed. Then automated histogram thresholding is applied to the masked Red channel pixels to segment foreground objects from the background and these foreground pixels are assigned to a mask labeled mask D.

[000132] At **206**, derive Brightness channel from Red, Blue, and Green channels.

[000133] At **207**, first the Brightness channel is masked with mask C and all pixels out of mask C are removed. Then automated histogram thresholding is applied to the masked Brightness channel pixels to segment foreground objects from the background and these foreground pixels are assigned to a mask labeled mask F.

[000134] At **208**, apply automated histogram thresholding to Green channel in the region of interest and save result to mask E.

[000135] At **209**, first a logical <OR> operation is applied to combine mask D with F and then a <XOR> is applied to mask with E, and the result is saved to mask G as the preliminary mask of fibers.

[000136] At **210**, as fine adjustment, within mask G remove pixels with energy in Red channel higher than energy in Green channel over certain proportion, and save the result to mask H, as the primary mask of fibers.

[000137] At **211**, first dilate and fill fiber circles in mask H, save result to J; then erode mask J to remove all thin non-scattered fibers that don't encircle, and save result to mask K.

[000138] At **212**, check whether or not mask K is empty. If K is not empty then continue to step **213**. Otherwise, jump to step **214**.

[000139] At **213**, count in mask K number of scattered thick fibers and number of filled circles formed by fibers. Grading result is based on the counted numbers and the grade is limited to the range of 3 to 4. Jump to step **216**.

[000140] At **214**, first count number of non-zero pixels in mask H and save result to variable x. **2**; then calculate logarithm of variable x with preset offsets, and save result to variable y.

[000141] At **215**, linearly map floating-point variable y to grades 0 – 4, as analysis result. The interval between two consecutive grades is 0.5.

[000142] At **216**, mask original image with mask G and display the masked image of fibers along with the grade value from prior step as numerical analysis result.

[000143] By applying image processing and analysis algorithms method **100** can display fibers in reticulin images as well as corresponding grading results. Method **100** can be implemented as either computer hardware or software or a combination of both, and can be implemented as a stand-alone program or a program combined with image sampling and preprocessing programs. Method **100** contains automated image segmentation and morphological processing, numerical image data analyzing, and user interfacing.

[000144] At **101**, an image subject as being analyzed in **100** is loaded into computer memory separated into three distinct channels labeled as Red, Blue and Green channels.

[000145] At **102**, automated histogram thresholding, such as ISODATA or Entropy thresholding, is applied to the Red channel pixels to segment foreground objects from the background and these foreground pixels are assigned to a mask labeled mask A.

[000146] At **103**, automated histogram thresholding, such as ISODATA or Entropy thresholding, is applied to the Green channel pixels to segment foreground objects from the background and these foreground pixels are assigned to a mask labeled mask B.

[000147] At **104**, a logical <OR> operation is applied to combine results of prior two steps, mask A and B, to get primary region of interest, as mask C.

[000148] At **105**, first the Red channel is masked with mask C and all pixels out of mask C are removed. Then automated histogram thresholding is applied to the masked Red channel pixels to segment foreground objects from the background and these foreground pixels are assigned to a mask labeled mask D.

[000149] At **106**, derive Brightness channel from Red, Blue, and Green channels.

[000150] At **107**, first the Brightness channel is masked with mask C and all pixels out of mask C are removed. Then automated histogram thresholding is applied to the

masked Brightness channel pixels to segment foreground objects from the background and these foreground pixels are assigned to a mask labeled mask F.

[000151] At **108**, apply automated histogram thresholding to Green channel in the region of interest and save result to mask E.

[000152] At **109**, first a logical <OR> operation is applied to combine mask D with F and then a <XOR> is applied to mask with E, and the result is saved to mask G as the preliminary mask of fibers.

[000153] At **110**, as fine adjustment, within mask G remove pixels with energy in Red channel higher than energy in Green channel over certain proportion, and save the result to mask H, as the primary mask of fibers.

[000154] At **111**, count number of non-zero pixels in mask H and save result to variable x.

[000155] At **112**, calculate logarithm of variable x with preset offsets, and save result to variable y.

[000156] At **113**, linearly map floating-point variable y to grades 0 – 4, as analysis result. The interval between two consecutive grades is 0.5.

[000157] At **114**, mask original image with mask G and display the masked image of fibers along with the grade value from prior step as numerical analysis result.

CONCLUSION

[000158] The invention provides an automated method of image analysis which determines reticulin fiber population statistic, in a greatly improved manner over manual scoring techniques and new and improved method over prior art in this field. By combining the scientific advantages of automation and the described method, as well as the greatly increased speed with which population can be evaluated, the invention is a major improvement over methods currently available.

[000159] The operator is provided with an option to configure the system to perform any or all of these steps and whether to perform certain steps more than once or several times in a row. The sequence of steps can be varied and thereby optimized for specific reagents or reagent combinations; however, the sequence described herein is preferred. An automated image analysis system identifies reticulin fibers in the context of

the bone marrow cellularity. The bone marrow cellularity is obtained using hematoxylin and eosin or PAS stain and uses a related program that is not included with this application. That bone marrow cellularity automated program is in patent application stage. In broader application of this invention, other cells could be jointly analyzed with the reticulin fibers including bone marrow cancer or tumor metastasis, nuclear stained cells, and other relevant immunohistochemically stained cells in combination with other patented tecnologies or commercially available technics.

Computer Implementation

[000160] Components of the invention may be realized in hardware or software, or a combination of both. However, preferably, the algorithms and methods of the invention are implemented in one or more processor programs executing on programmable computers each comprising at least one processor of either Intel or Motorola type, at least one data storage system (including volatile and non-volatile memory and/or storage elements), at least one input device, and at least one output device. Program code(s) applied to input data may perform the functions described herein to generate desired output information. The output information is applied to one or more output devices, in known fashion but preferably either an Excel compatible format or a graphics plot showing the cells and the reticulin fibers. Each program may be implemented in any desired computer language preferably with high level image processing functions (including machine, assembly, high level procedural, or object oriented programming languages) to communicate with a computer system. In any case, the language may be a compiled or interpreted language or both. Each such computer program is preferably stored on a storage media or device (e.g., ROM, CD-ROM, DVD, tape, or magnetic diskette) readable by a general or special purpose programmable computer, for configuring and operating the computer when the storage media or device is read by the computer to perform the procedures described herein. The inventive system may also be considered to be realized as a computer-readable storage medium, or via an internet server medium and hardware configured with connectivity and said computer program, where the storage medium so configured causes a computer to operate in a specific and predefined manner to perform the functions described herein.

ADDITIONAL EMBODIMENTS

[000161] There are a plurality of embodiments including a provision for incorporation of other modules to include bone marrow cellularity and immunohistochemistry with nuclear reactivity, membrane-cytoplasmic cellular stains, spindle shaped or vessel staining, global stromal collagen staining pattern and others. Another embodiment is to display not grade but other distribution or statistical characteristics such as length, coarse ness, fiber diameters, crossovers of fibers, intensity and relation to other cells like megakaryocytes, blood vessels, and tumor cell infiltrates. An ability to include and exclude areas in the bone marrow for analysis is also an embodiment.

[000162] Another important embodiment is the capability of the algorithm to convert between the 0 to 4 grades and the 0 to 3 grades by manually transposing the definitions of normal, mild, moderate, and severe. In the 0 to 4 grading system, the 0 to 2 is equivalent to 0 of the Thiele system, the 2 to 3 is equal to 2, the 3 to 4 is equal to the

Thiele grade 3. A training system using the Gomori staining method of Thiele is easily implemented using the basic algorithm methods and is within the capability of the adaptive algorithm.

[000163] Another preferred embodiment is the capability of the apparatus and method to be used in ordinary daily pathology practice setting where the diagnostic process of tissue biopsy is the priority. In this mode, the user has little time fiddling with controls of the system and therefore require a robust accommodating mode. In this embodiment, the system is made to dynamically accommodate a wide variation of microscope transmitted light intensity from 4.0 to 6.6 variable rheostat setting (0-10 range).

[000164] Another preferred embodiment is the capability of the invention to accommodate a variable technics including reticulin using manual stain, or using automated machines such as Ventana NEXES stainers or other slide products performed by different automated immunohistochemistry machines.

[000165] Another preferred embodiment is the capability of the invention to accommodate variable counterstains, both as indicia marker or nuclear counter stain, to include not only black, red or brown but a combination of other colored dyes.

[000166] In one embodiment, examined for speed, a segmentation of a 512.times.474 RGB image and display of statistical results table or plot data result in a 12-15 seconds using a 100 MHz PowerPC CPU or faster in a Intel Celeron PC 1.4 GHZ using JAVA executed the method in 2-3 seconds.

[000167] The invention may be embodied in other forms than those specifically disclosed herein without departing from its spirit or essential characteristics. The described embodiments are to be considered in all respects as illustrative and not restrictive. Since numerous modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and operation shown and described, and accordingly, all suitable modifications and equivalents may be resorted to, falling within the scope of the invention.

DRAWINGS

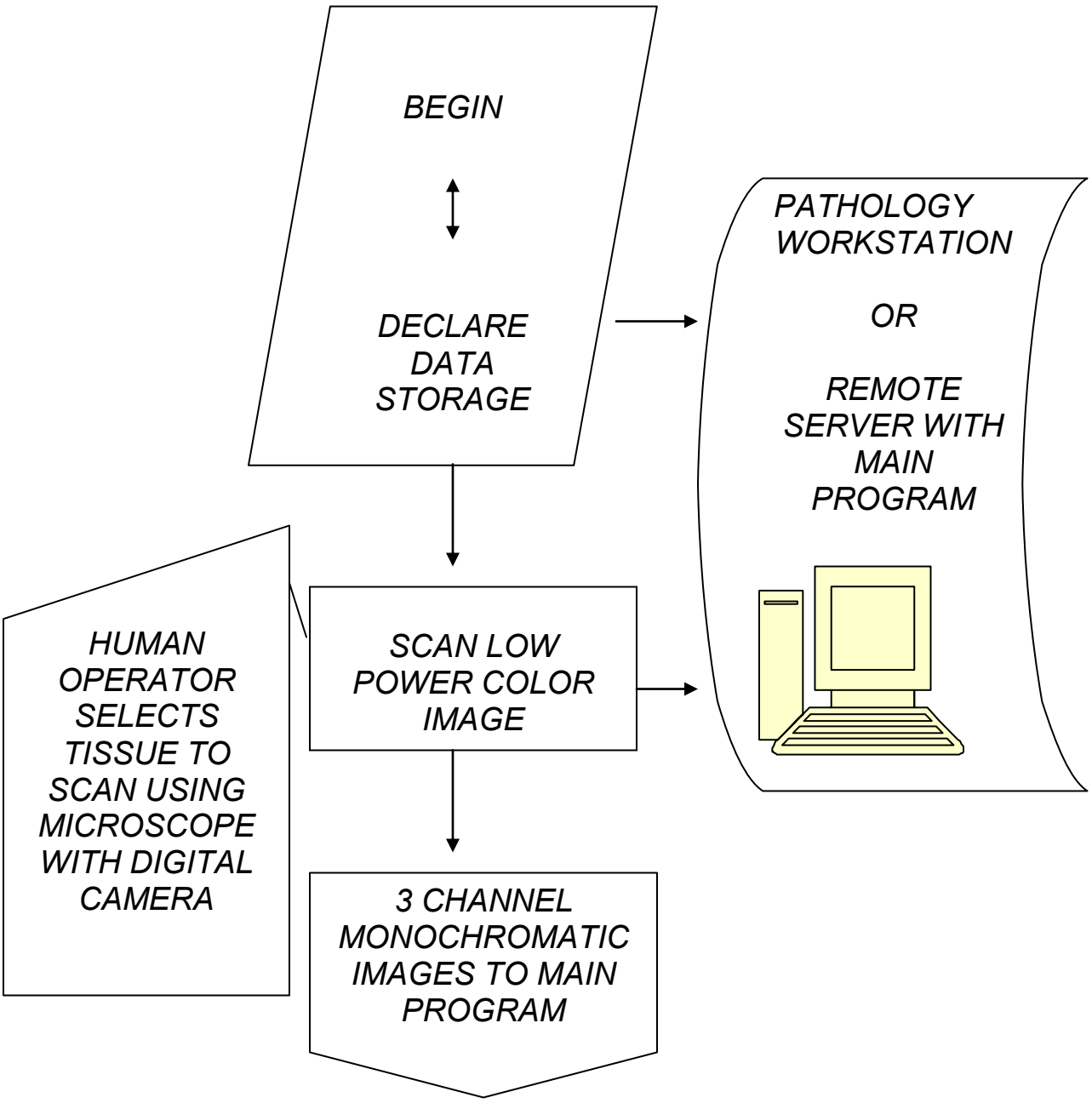

FIG. 1

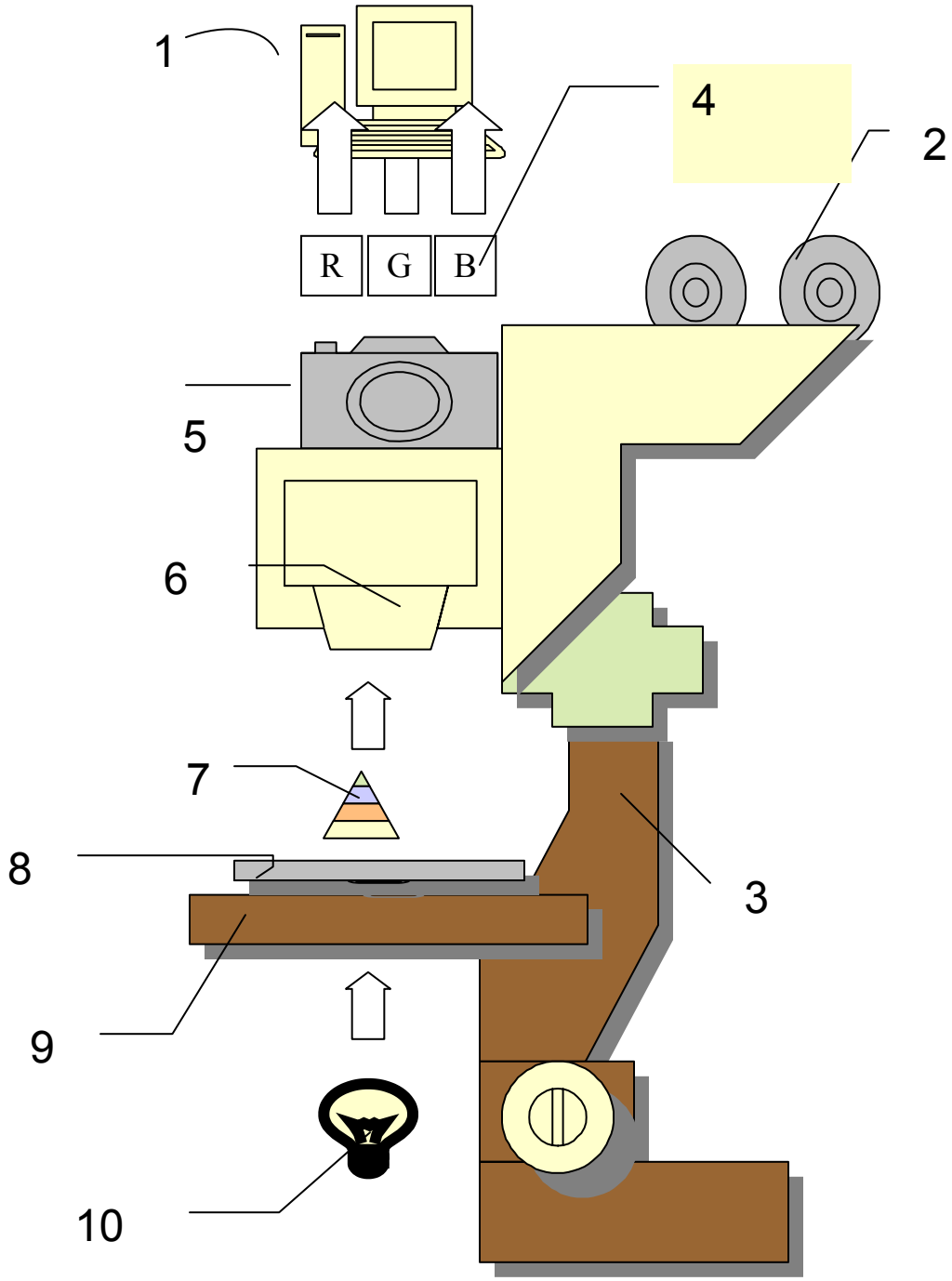

FIG. 2

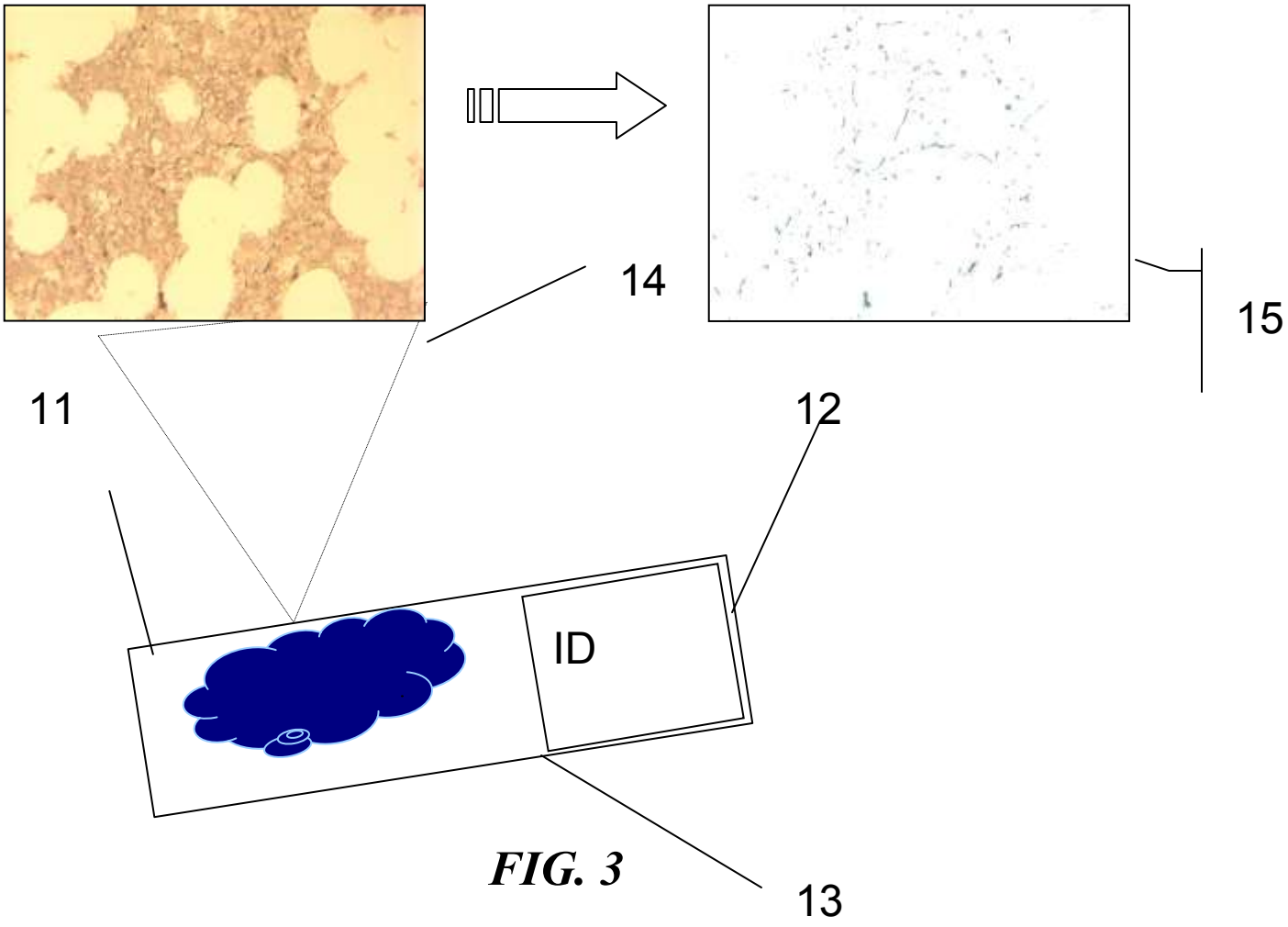

FIG. 3

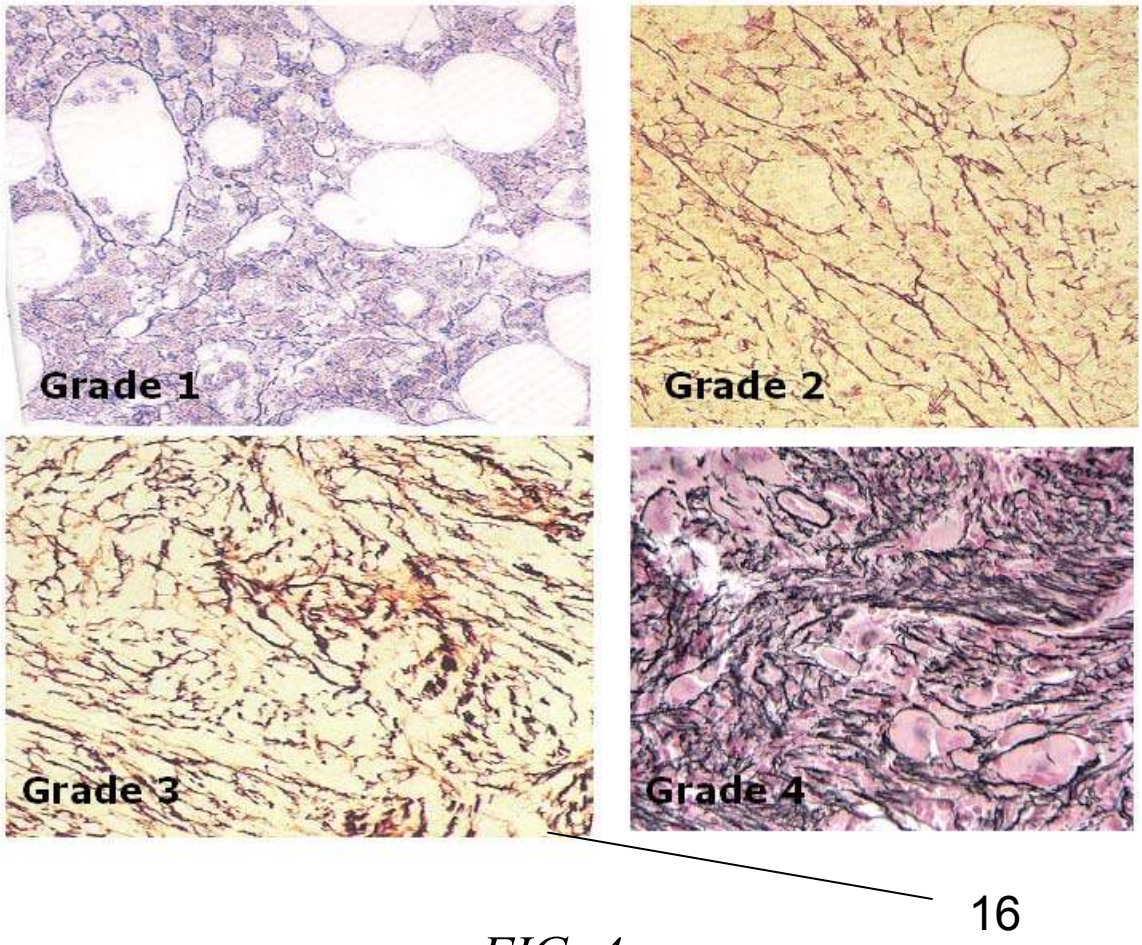

FIG. 4a

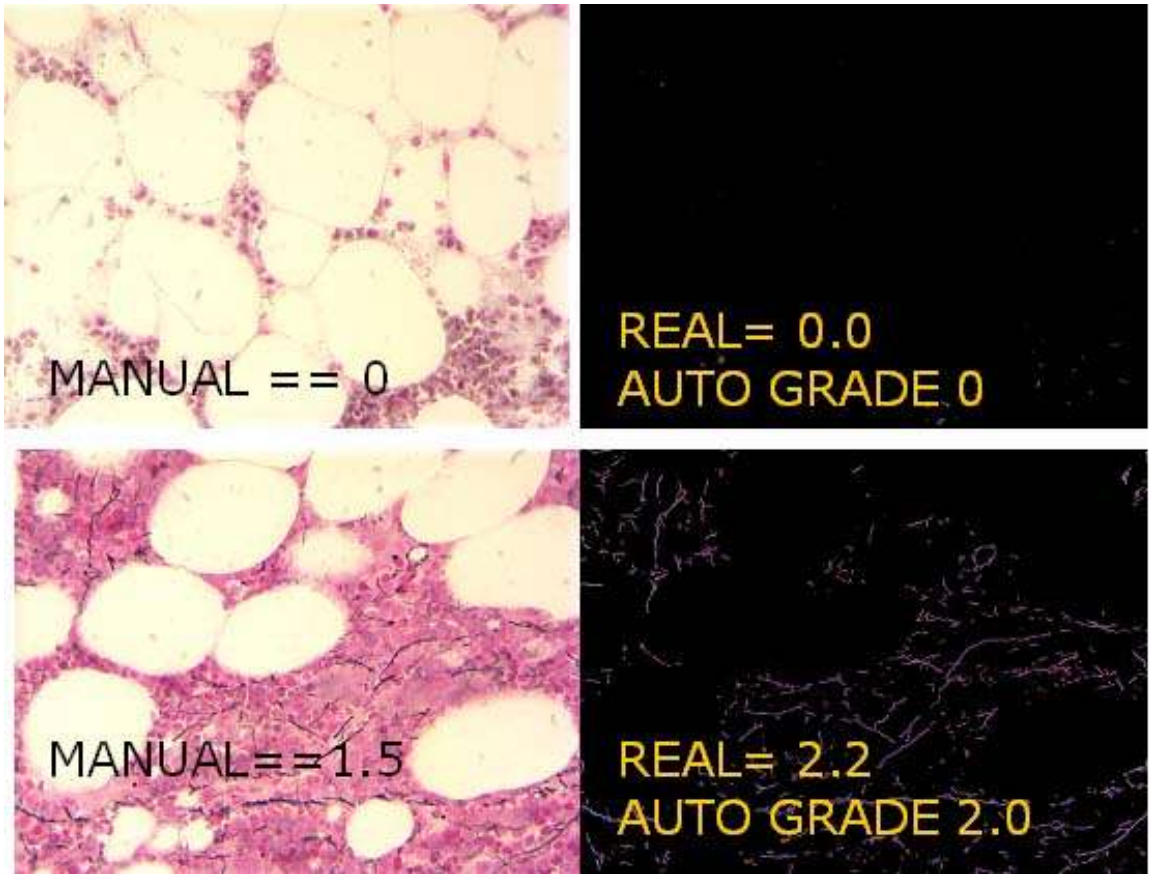

Fig 4b

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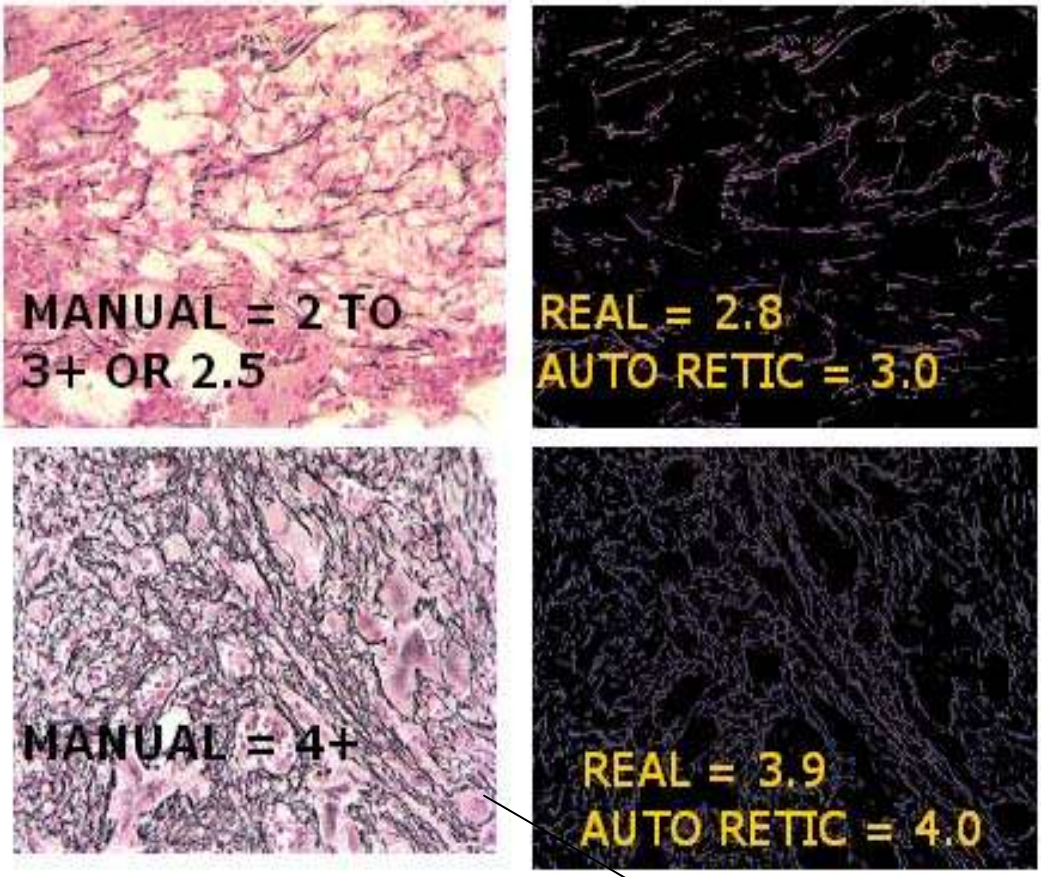
MANUAL = 2 TO
3+ OR 2.5
REAL = 2.8
AUTO RETIC = 3.0
MANUAL = 4+
REAL = 3.9
AUTO RETIC = 4.0
18

Figure 4c

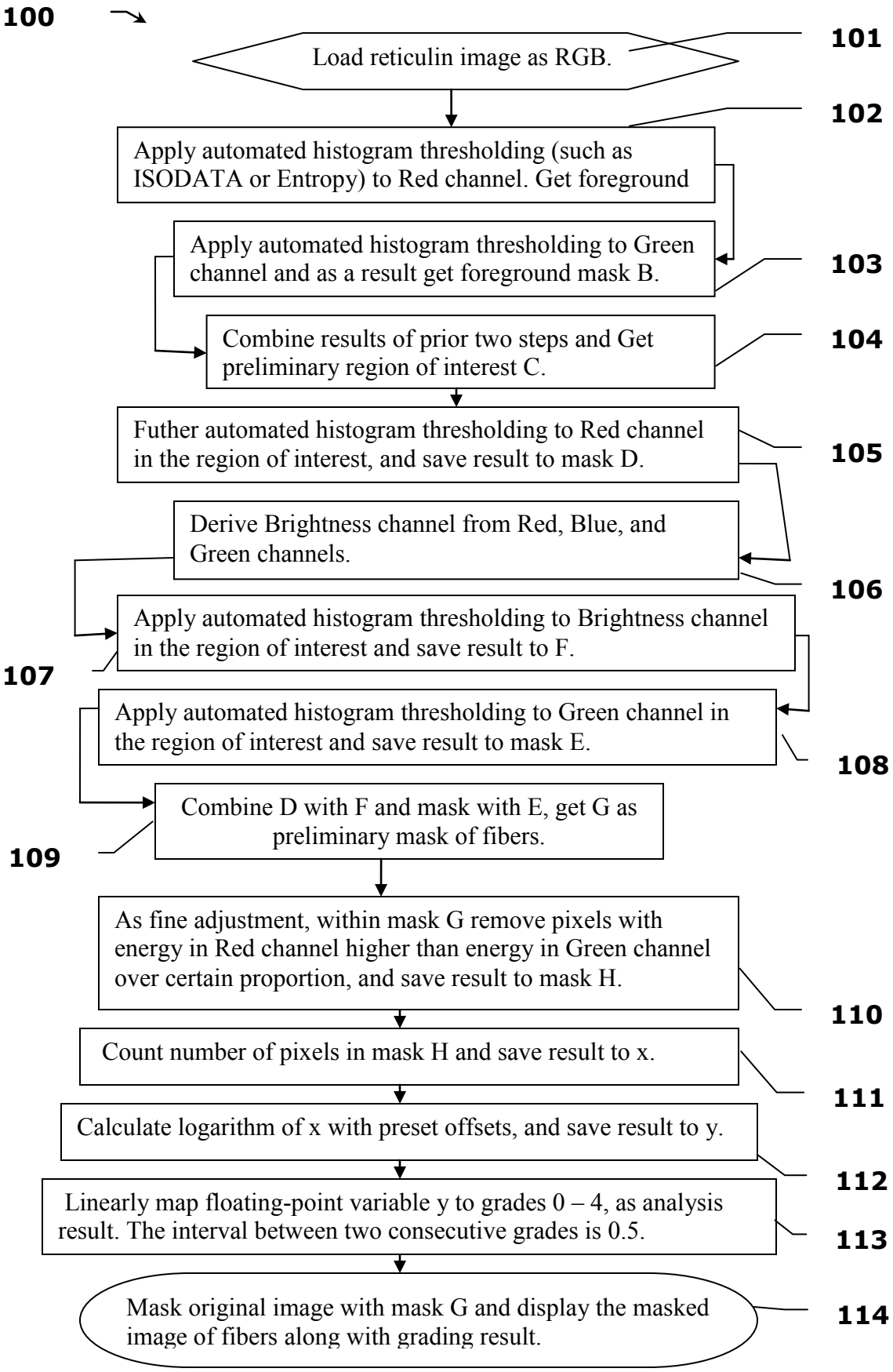

FIG.100 Grading Algorithm 1

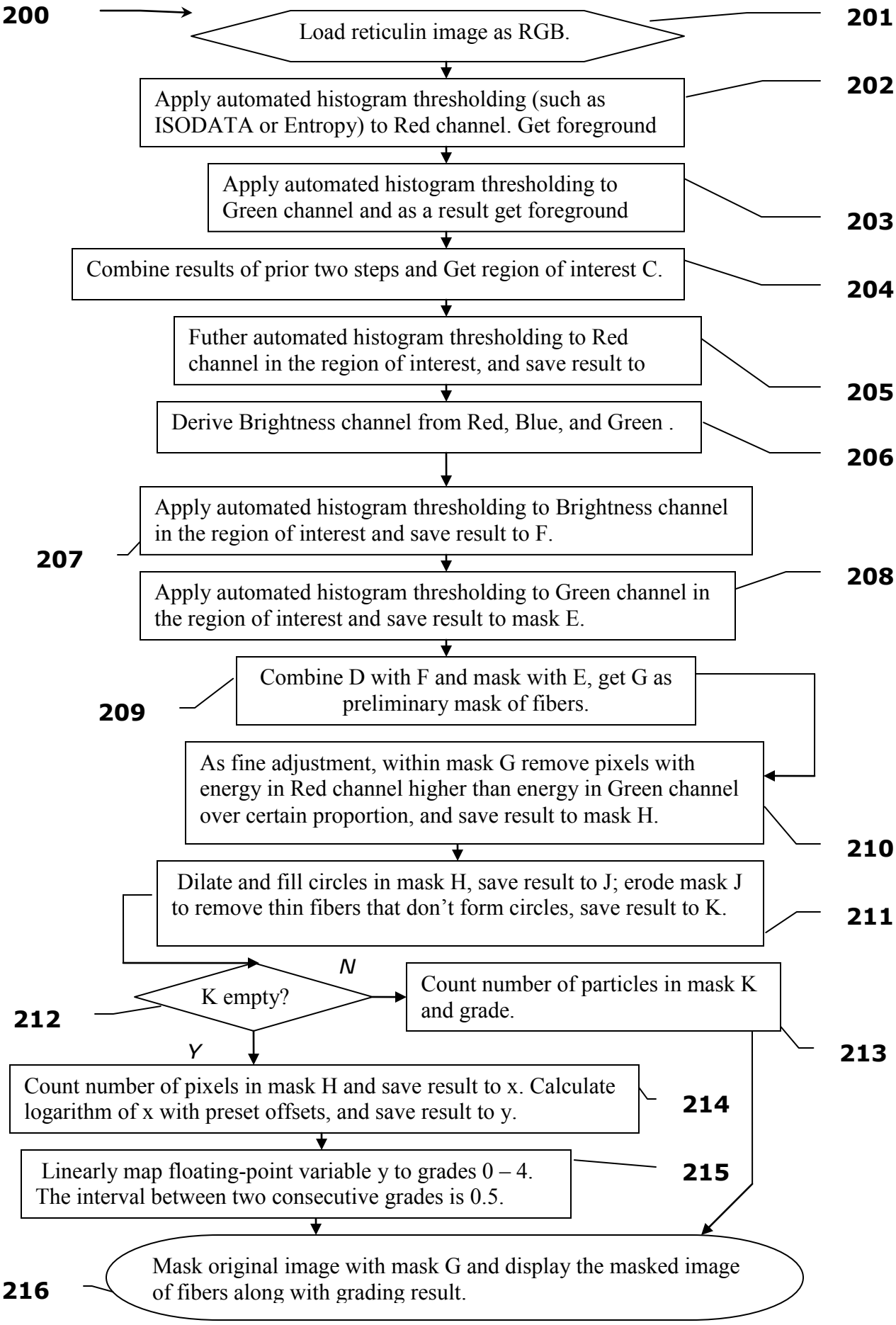

FIG.200 Grading Algorithm 2