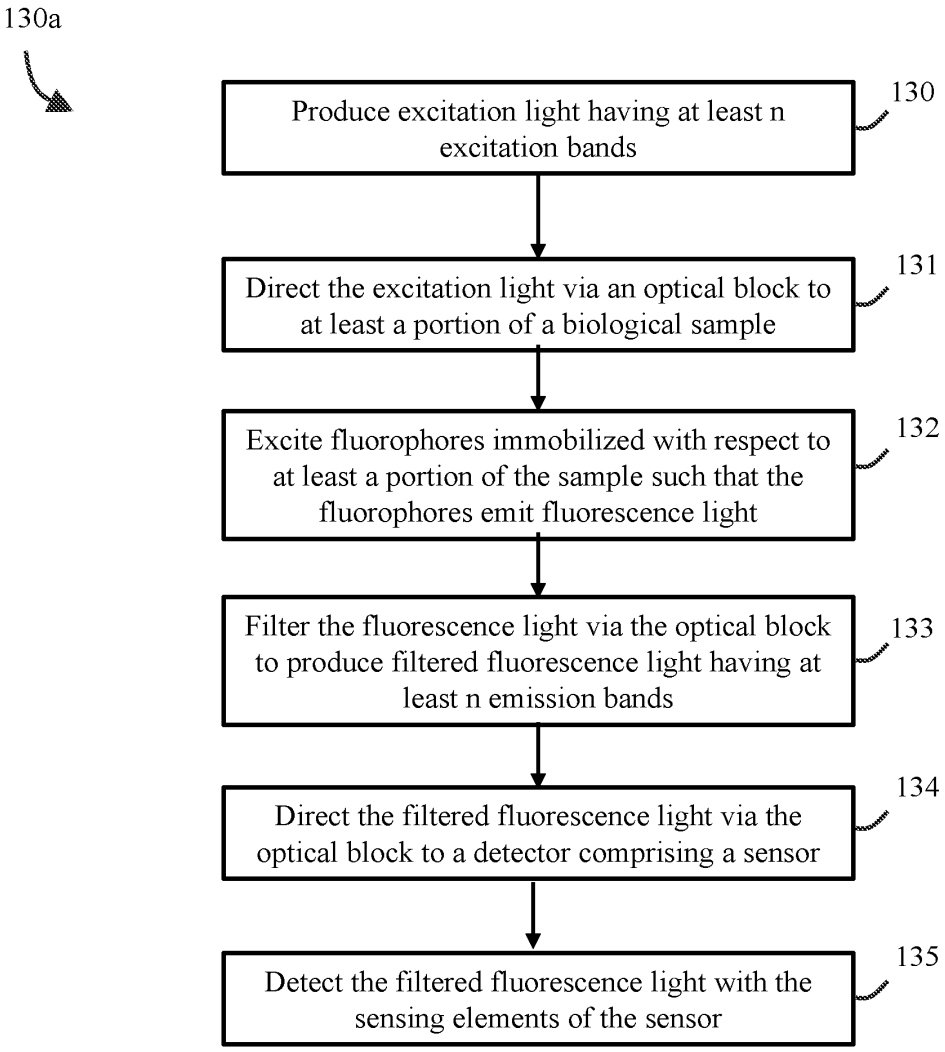

130a
Produce excitation light having at least n excitation bands
130
Direct the excitation light via an optical block to at least a portion of a biological sample
131
Excite fluorophores immobilized with respect to at least a portion of the sample such that the fluorophores emit fluorescence light
132
Filter the fluorescence light via the optical block to produce filtered fluorescence light having at least n emission bands
133
Direct the filtered fluorescence light via the optical block to a detector comprising a sensor
134
Detect the filtered fluorescence light with the sensing elements of the sensor
135

FIG. 1A

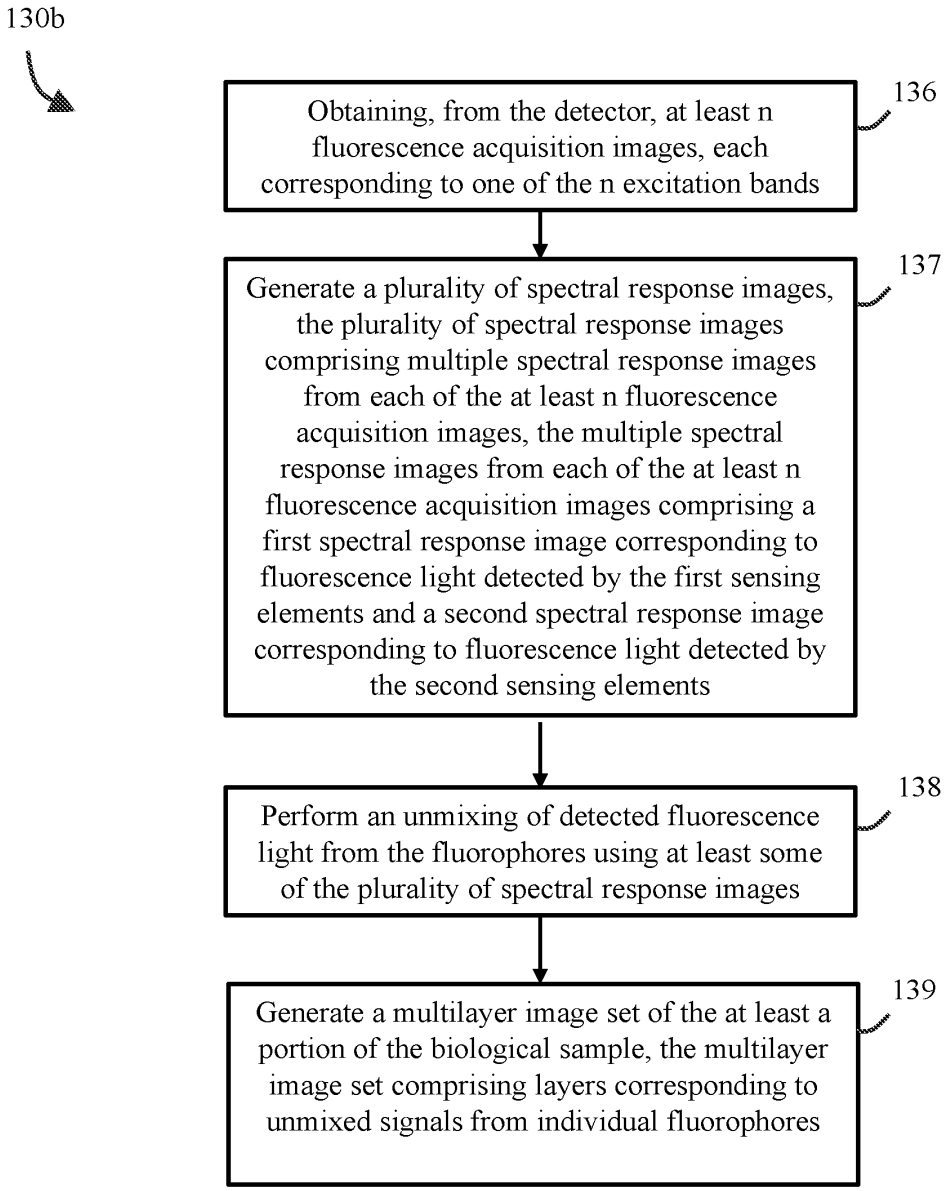

130b
Obtaining, from the detector, at least n fluorescence acquisition images, each corresponding to one of the n excitation bands
136
Generate a plurality of spectral response images, the plurality of spectral response images comprising multiple spectral response images from each of the at least n fluorescence acquisition images, the multiple spectral response images from each of the at least n fluorescence acquisition images comprising a first spectral response image corresponding to fluorescence light detected by the first sensing elements and a second spectral response image corresponding to fluorescence light detected by the second sensing elements
137
Perform an unmixing of detected fluorescence light from the fluorophores using at least some of the plurality of spectral response images
138
Generate a multilayer image set of the at least a portion of the biological sample, the multilayer image set comprising layers corresponding to unmixed signals from individual fluorophores
139

FIG. 1B

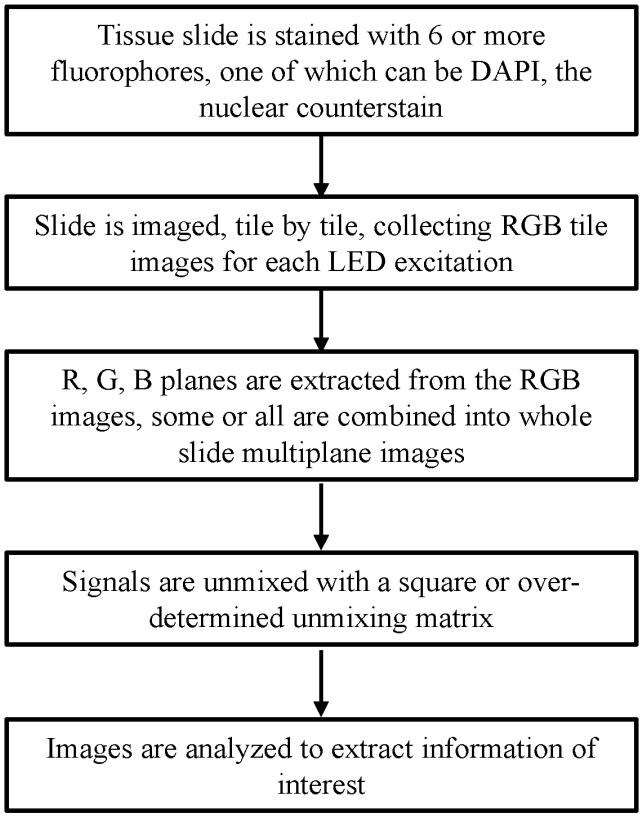

Tissue slide is stained with 6 or more fluorophores, one of which can be DAPI, the nuclear counterstain
Slide is imaged, tile by tile, collecting RGB tile images for each LED excitation
R, G, B planes are extracted from the RGB images, some or all are combined into whole slide multiplane images
Signals are unmixed with a square or over-determined unmixing matrix
Images are analyzed to extract information of interest

FIG. 1C

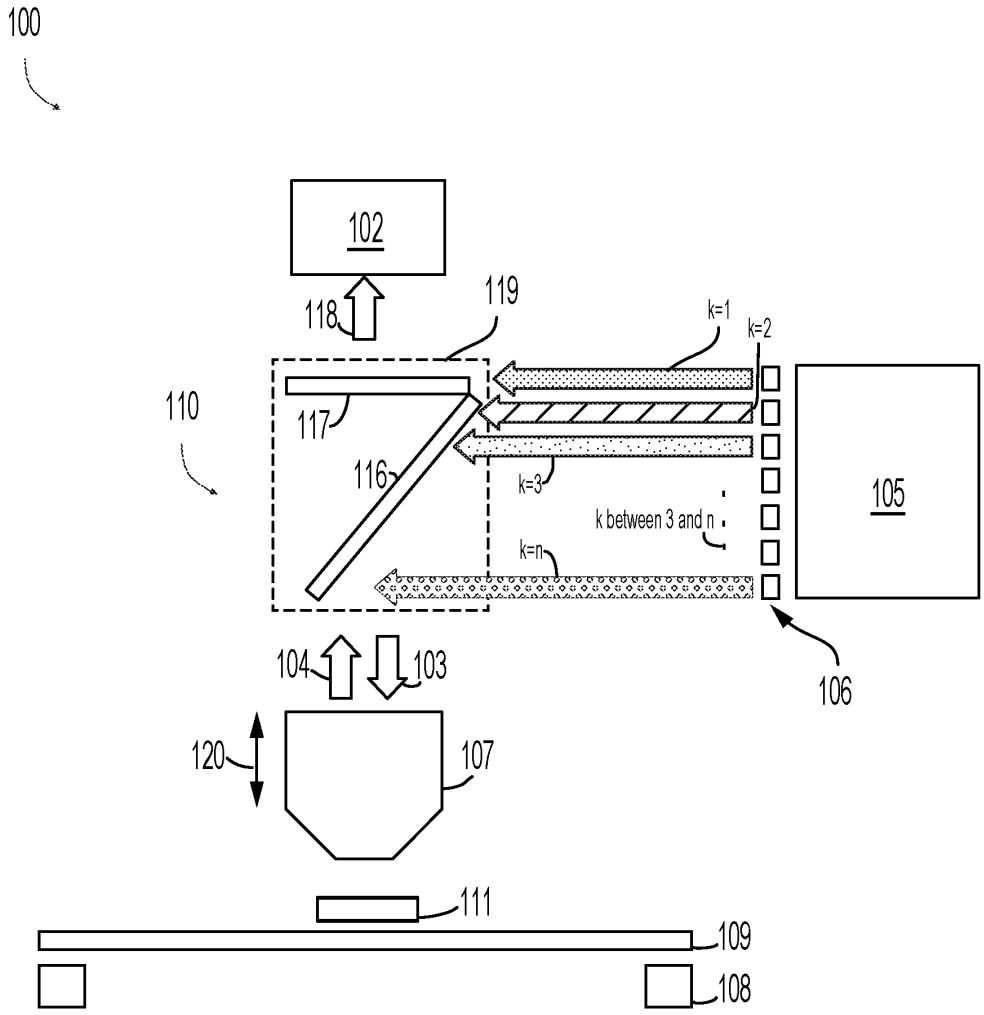

100
102
118
119
k=1
k=2
110
117
116
k=3
k between 3 and n
k=n
105
104
103
106
120
107
111
109
108

FIG. 2A

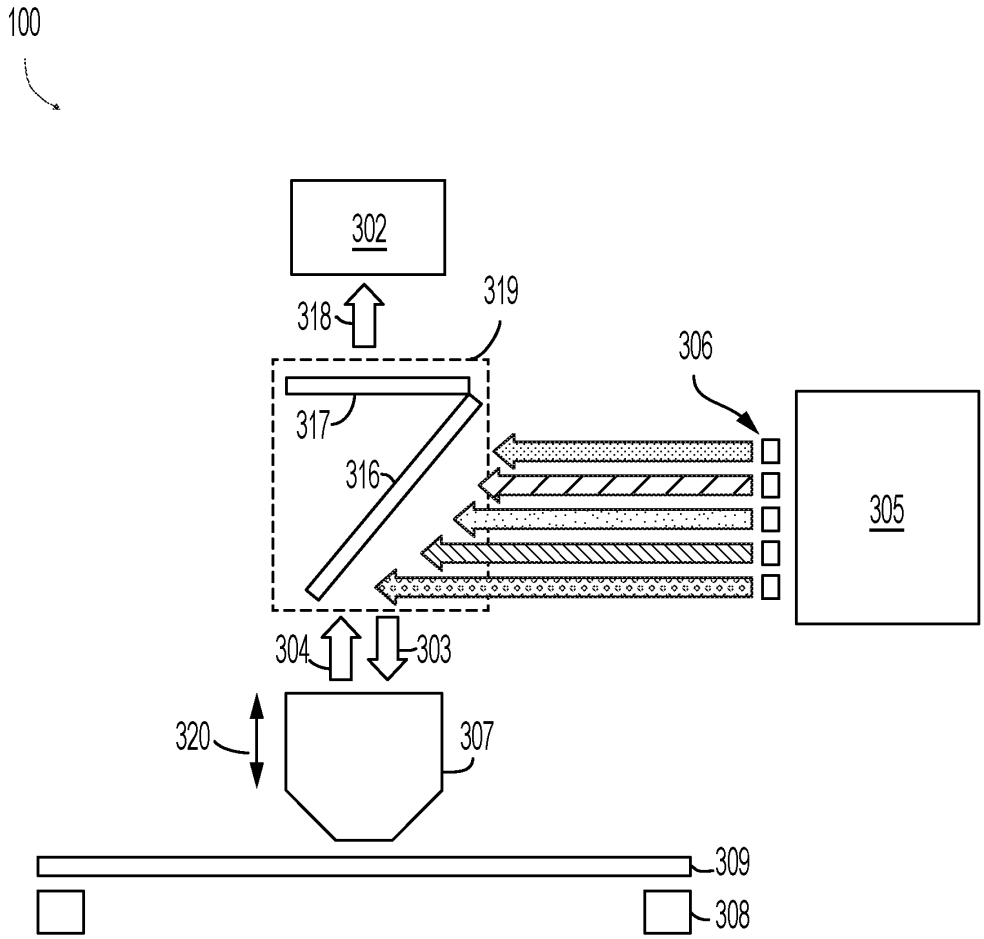


FIG. 2B

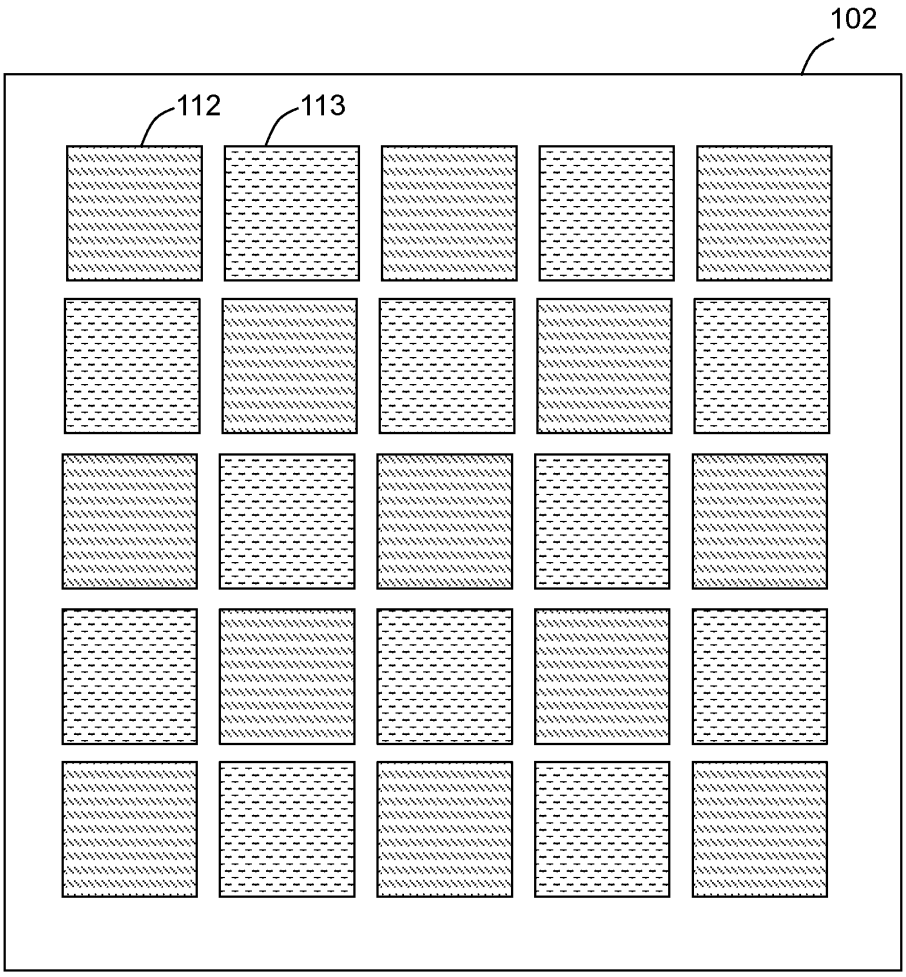


FIG. 2C

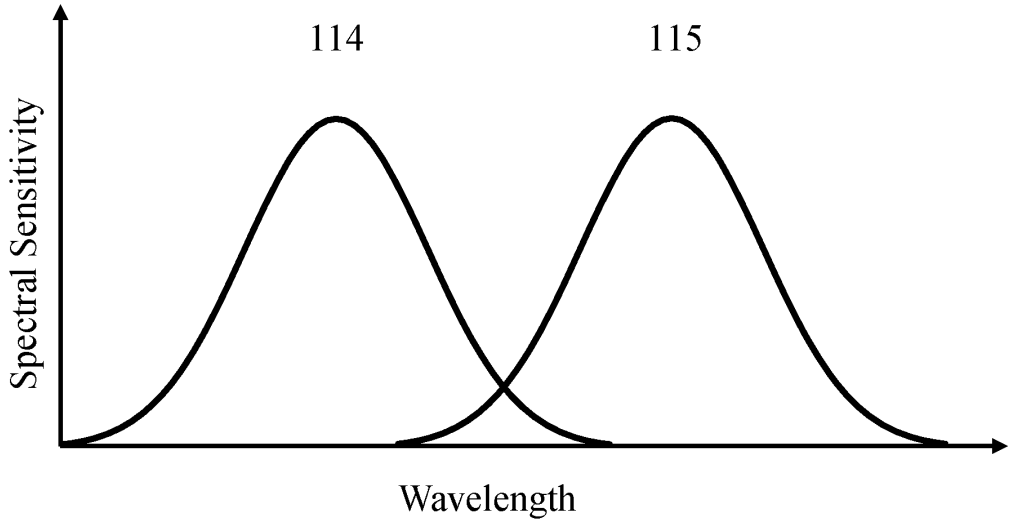


FIG. 2D

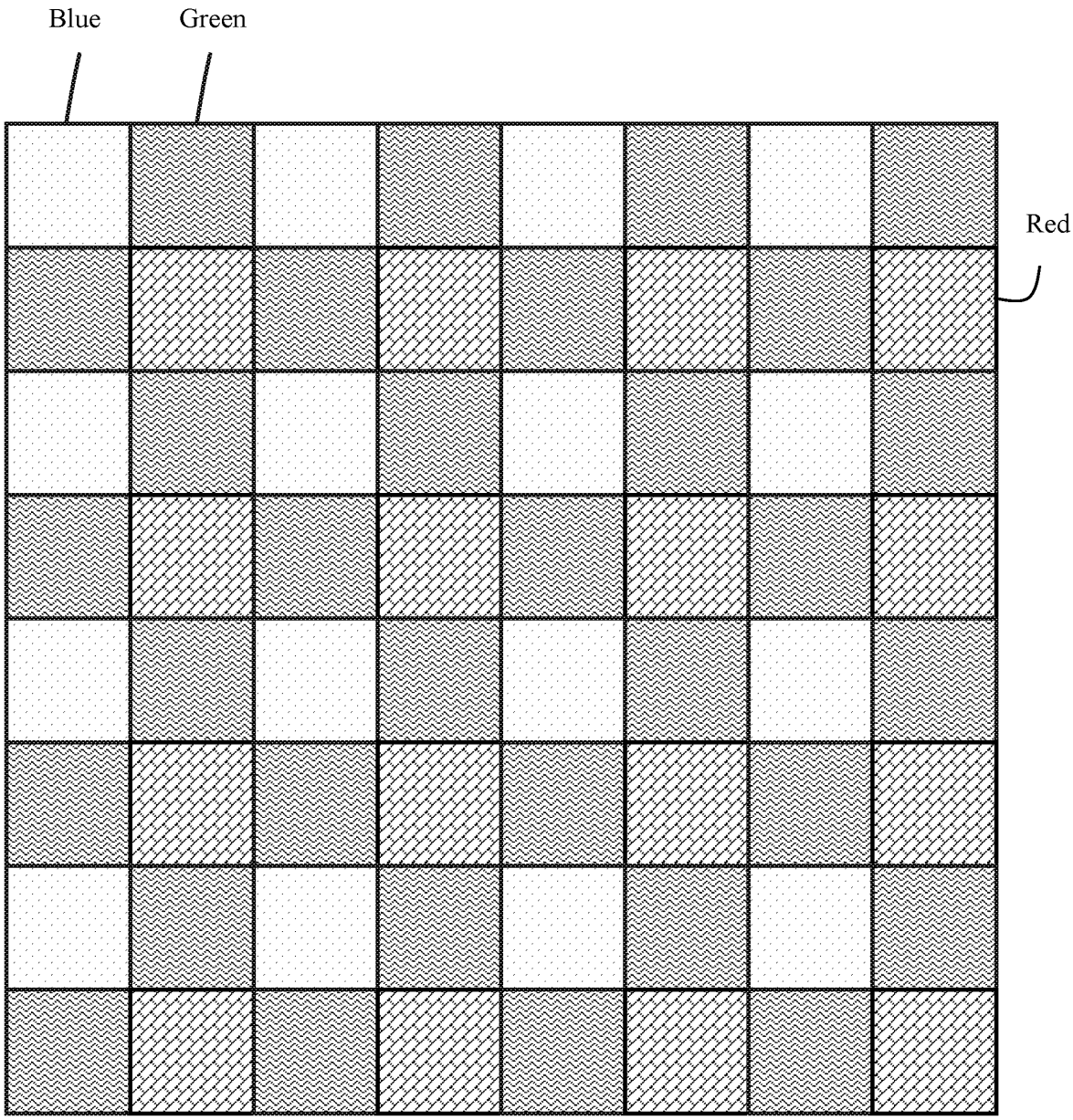


FIG. 2E

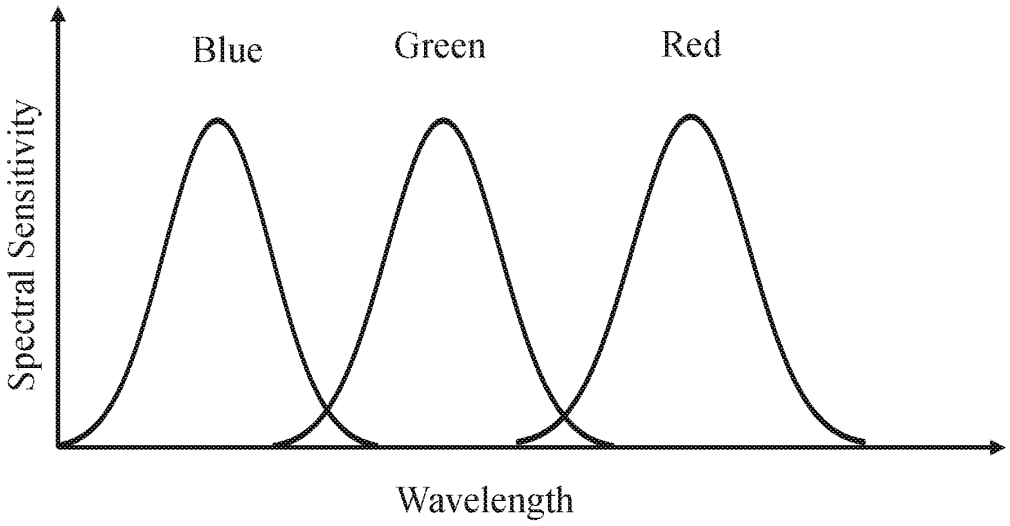


FIG. 2F

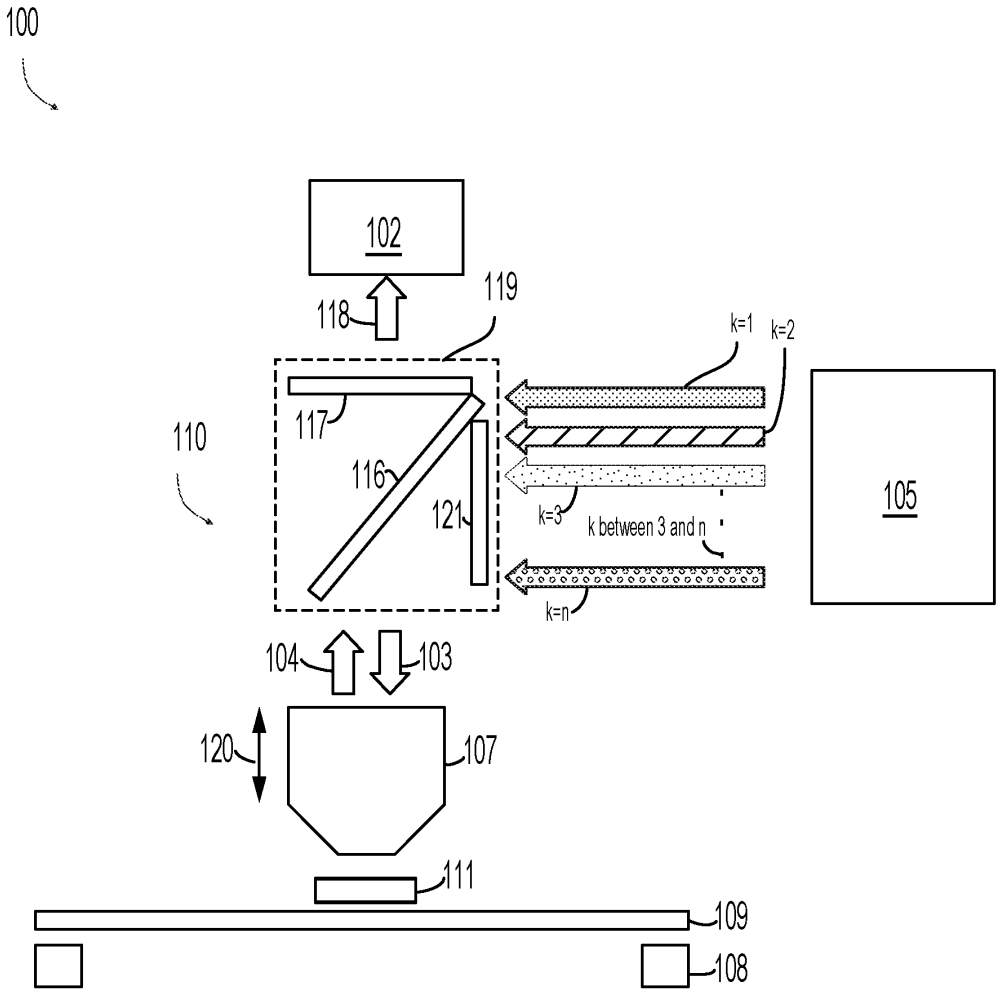

100
102
118
119
k=1
k=2
110
117
116
121
105
k=3
k between 3 and n
k=n
104
103
120
107
111
109
108

FIG. 3A

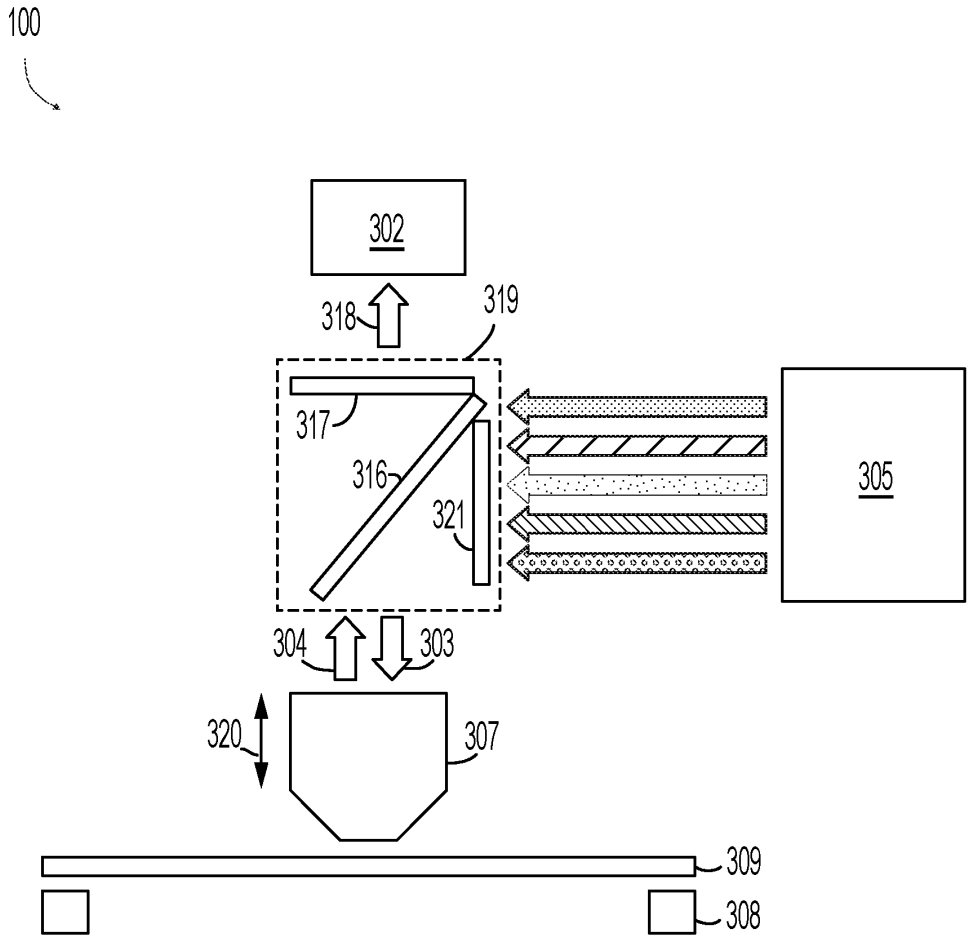


FIG. 3B

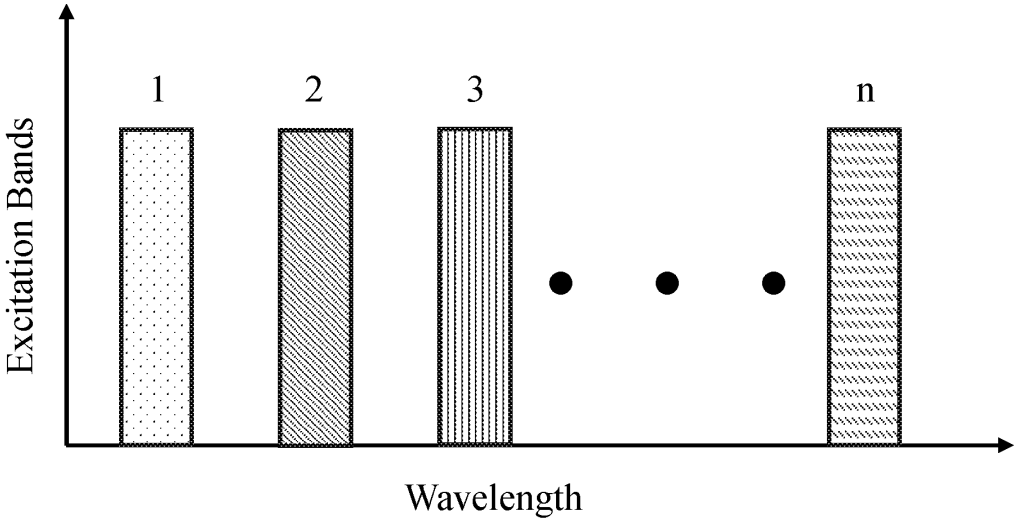

Excitation Bands
1
2
3
n
Wavelength

FIG. 3C

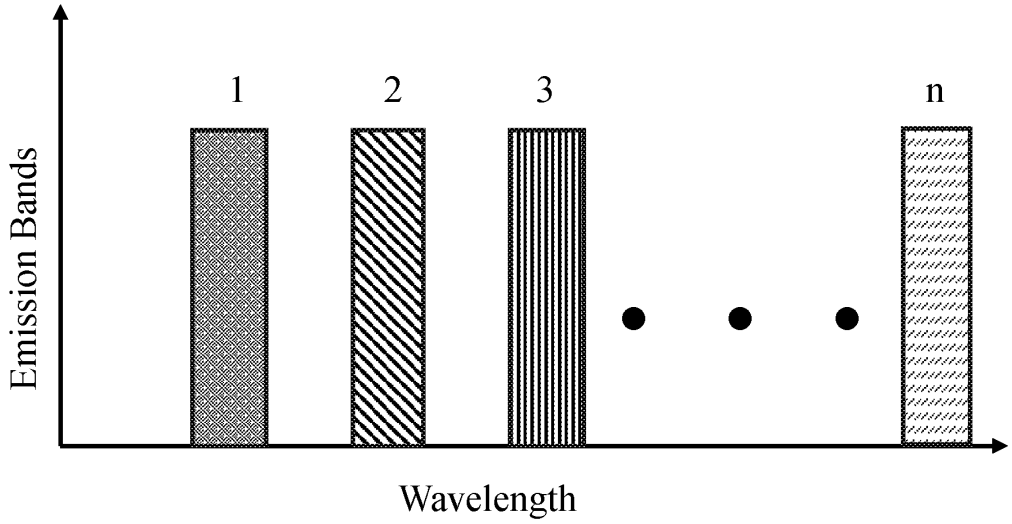

Emission Bands
1
2
3
n
Wavelength

FIG. 3D

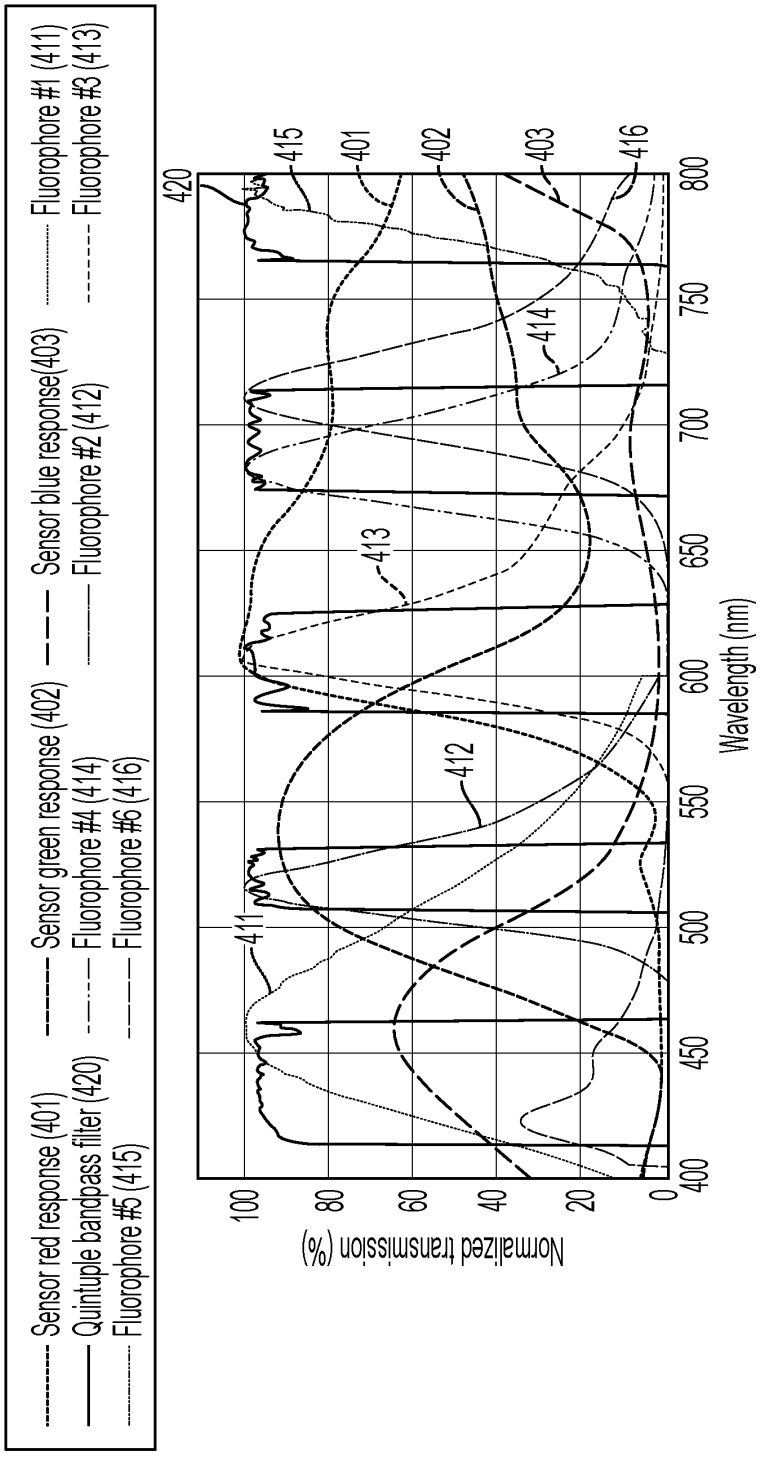
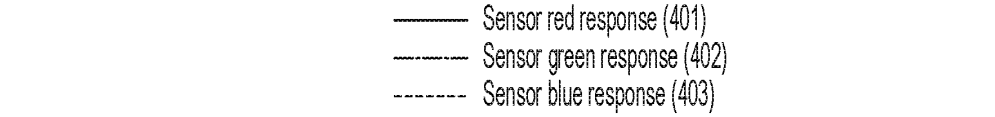

Sensor red response (401)
Sensor green response (402)
Sensor blue response(403)
Fluorophore #1 (411)
Quintuple bandpass filter (420)
Fluorophore #4 (414)
Fluorophore #2 (412)
Fluorophore #3 (413)
Fluorophore #5 (415)
Fluorophore #6 (416)
Normalized transmission (%)
Wavelength (nm)
0
20
40
60
80
100
400
450
500
550
600
650
700
750
800
411
412
413
414
415
416
401
402
403
420

FIG. 4



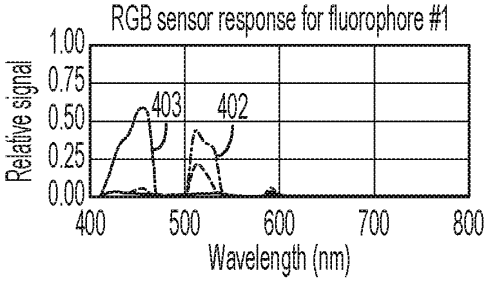


FIG. 5A

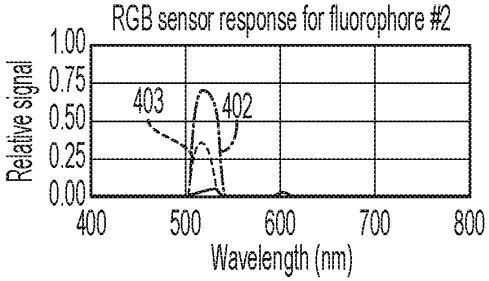


FIG. 5B

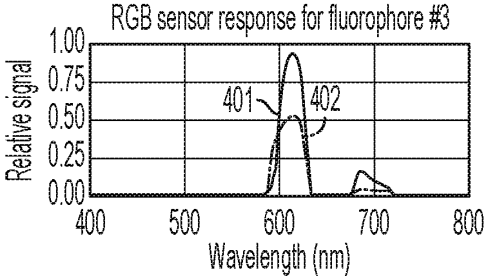


FIG. 5C

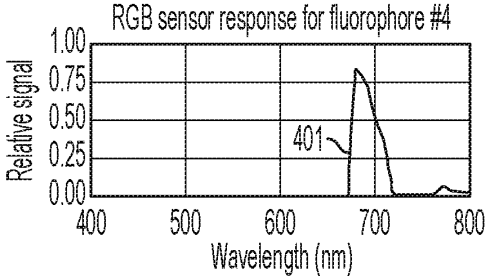


FIG. 5D

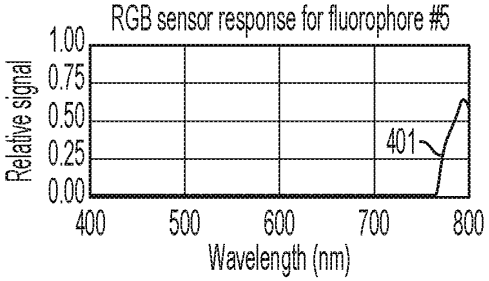


FIG. 5E

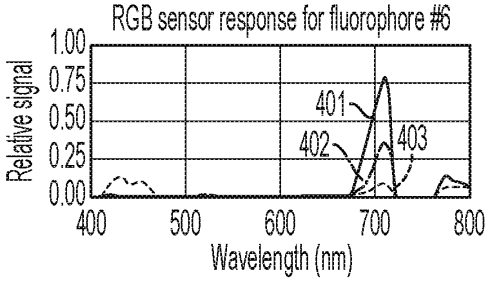


FIG. 5F

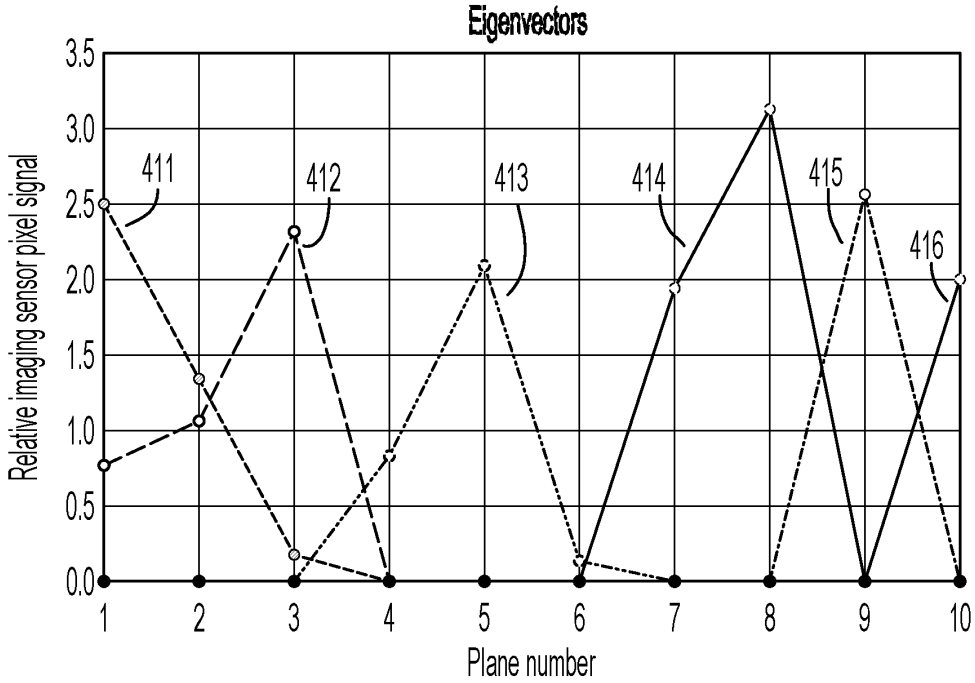

Eigenvectors
Relative imaging sensor pixel signal
Plane number
411
412
413
414
415
416
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
9
10

FIG. 6

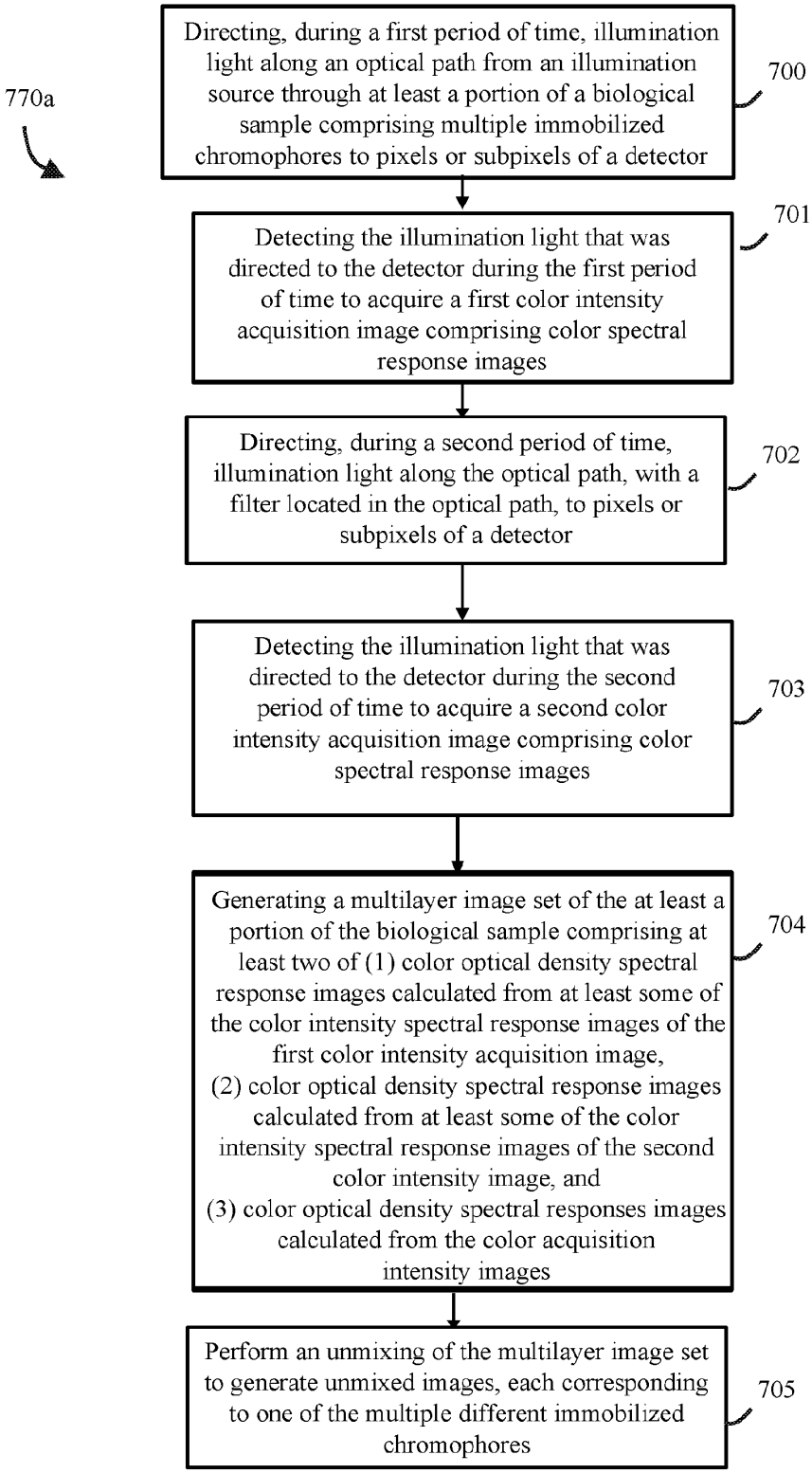

770a
Directing, during a first period of time, illumination light along an optical path from an illumination source through at least a portion of a biological sample comprising multiple immobilized chromophores to pixels or subpixels of a detector
700
Detecting the illumination light that was directed to the detector during the first period of time to acquire a first color intensity acquisition image comprising color spectral response images
701
Directing, during a second period of time, illumination light along the optical path, with a filter located in the optical path, to pixels or subpixels of a detector
702
Detecting the illumination light that was directed to the detector during the second period of time to acquire a second color intensity acquisition image comprising color spectral response images
703
Generating a multilayer image set of the at least a portion of the biological sample comprising at least two of (1) color optical density spectral response images calculated from at least some of the color intensity spectral response images of the first color intensity acquisition image,
(2) color optical density spectral response images calculated from at least some of the color intensity spectral response images of the second color intensity image, and
(3) color optical density spectral responses images calculated from the color acquisition intensity images
704
Perform an unmixing of the multilayer image set to generate unmixed images, each corresponding to one of the multiple different immobilized chromophores
705

FIG. 7A

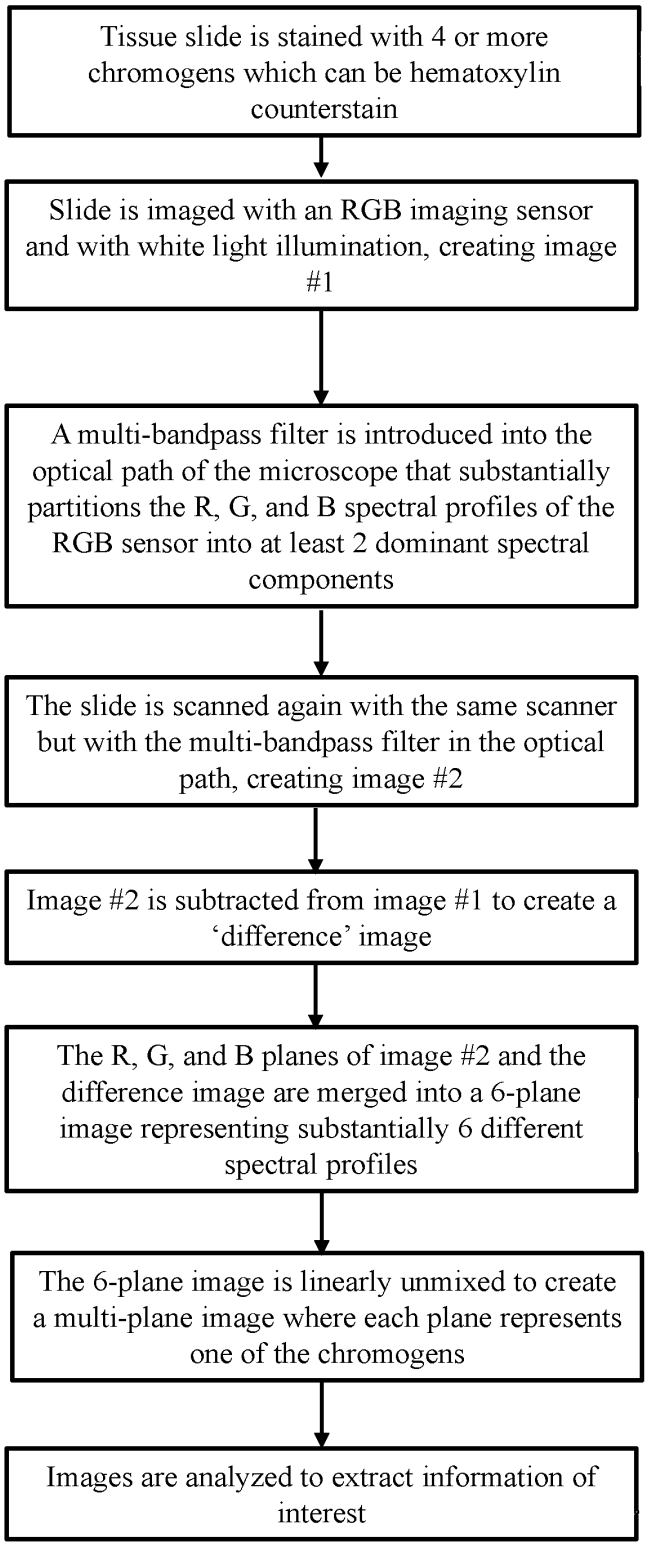

Tissue slide is stained with 4 or more chromogens which can be hematoxylin counterstain
Slide is imaged with an RGB imaging sensor and with white light illumination, creating image #1
A multi-bandpass filter is introduced into the optical path of the microscope that substantially partitions the R, G, and B spectral profiles of the RGB sensor into at least 2 dominant spectral components
The slide is scanned again with the same scanner but with the multi-bandpass filter in the optical path, creating image #2
Image #2 is subtracted from image #1 to create a 'difference' image
The R, G, and B planes of image #2 and the difference image are merged into a 6-plane image representing substantially 6 different spectral profiles
The 6-plane image is linearly unmixed to create a multi-plane image where each plane represents one of the chromogens
Images are analyzed to extract information of interest

FIG. 7B

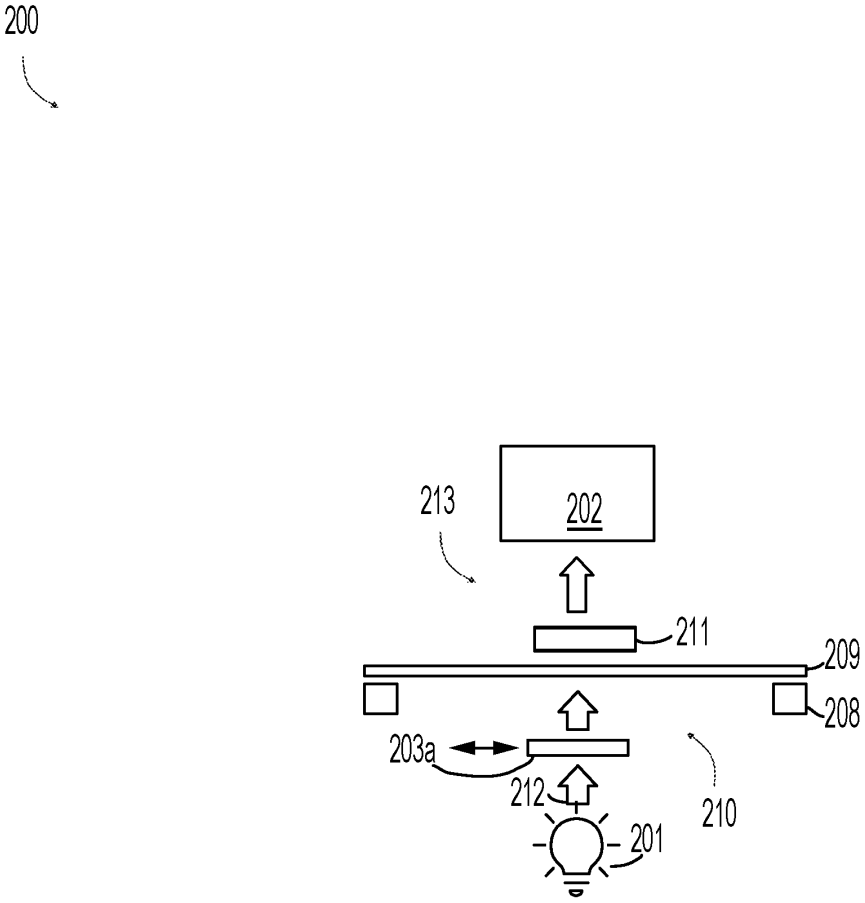


FIG. 8A

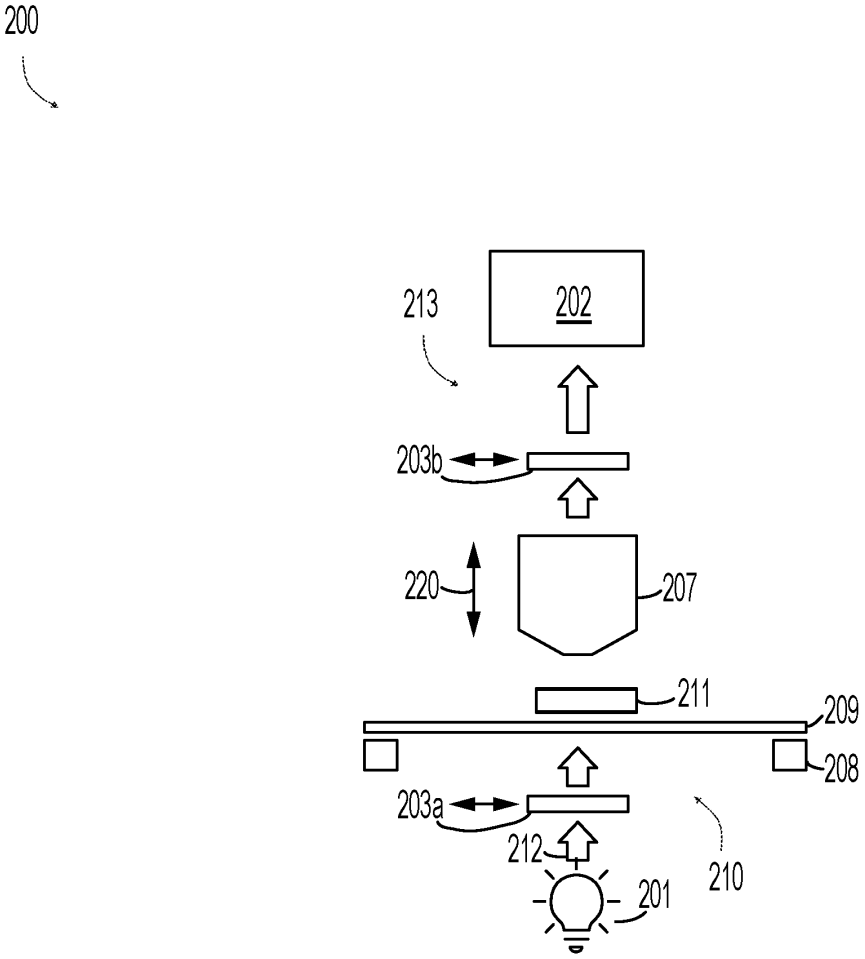


FIG. 8B

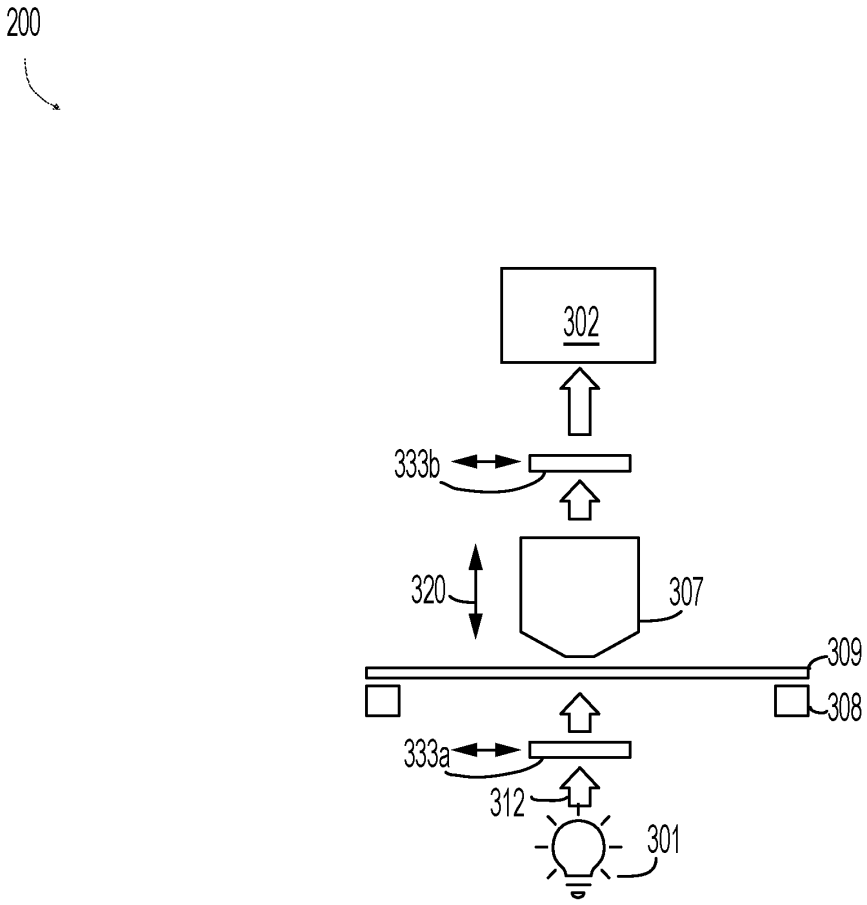


FIG. 8C

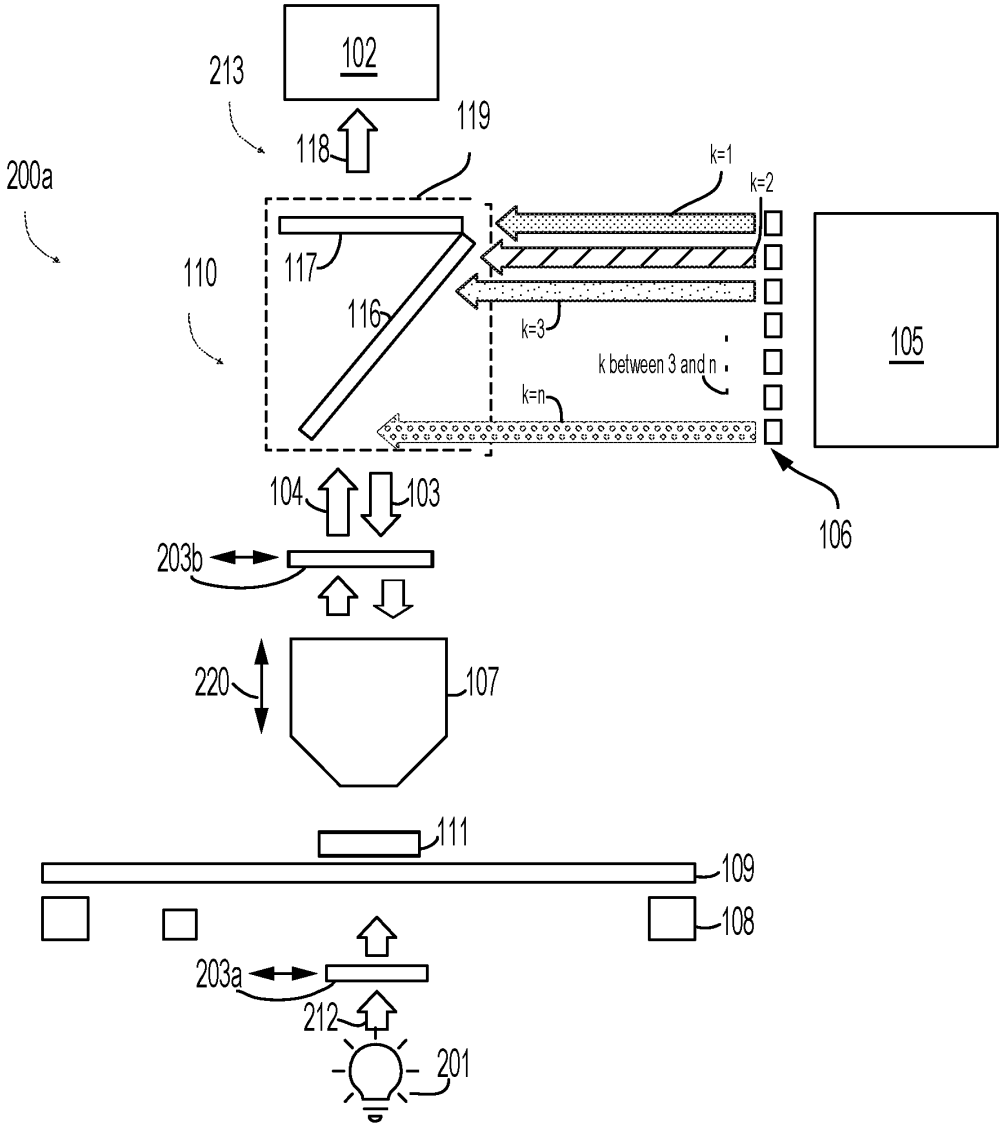
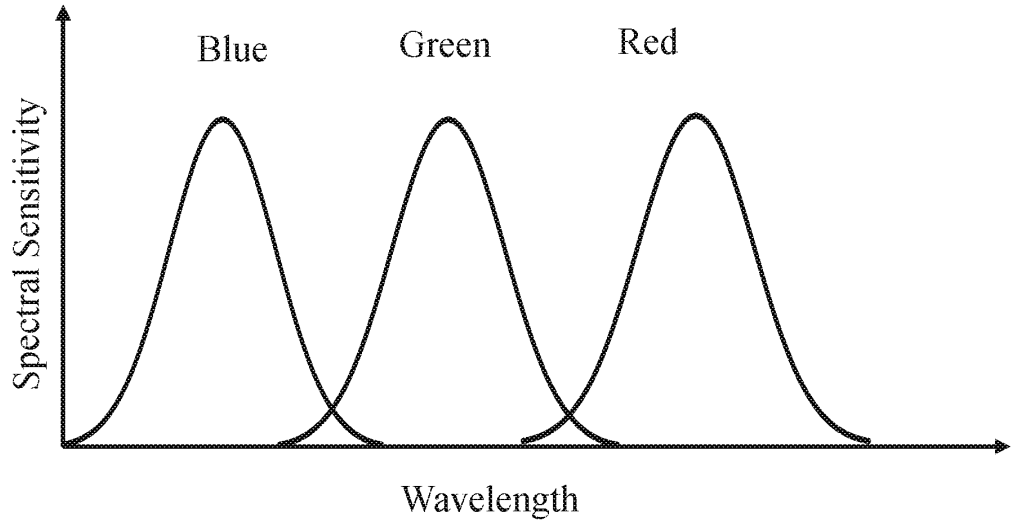

102
213
119
118
200a
k=1
k=2
110
117
116
k=3
105
k between 3 and n
k=n
104
103
106
203b
220
107
111
109
108
203a
212
201

FIG. 8D


Blue
Green
Red
Spectral Sensitivity
Wavelength

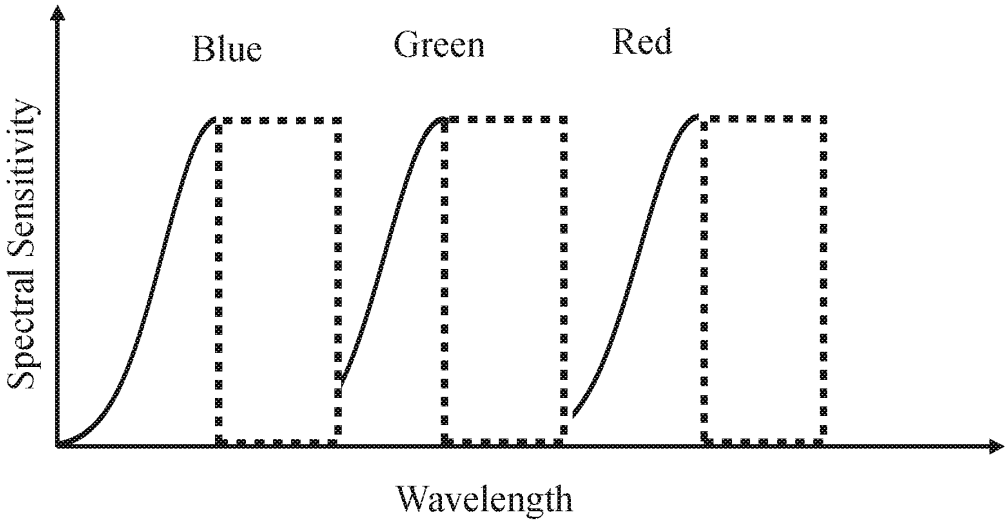

Blue
Green
Red
Spectral Sensitivity
Wavelength

FIG. 8E

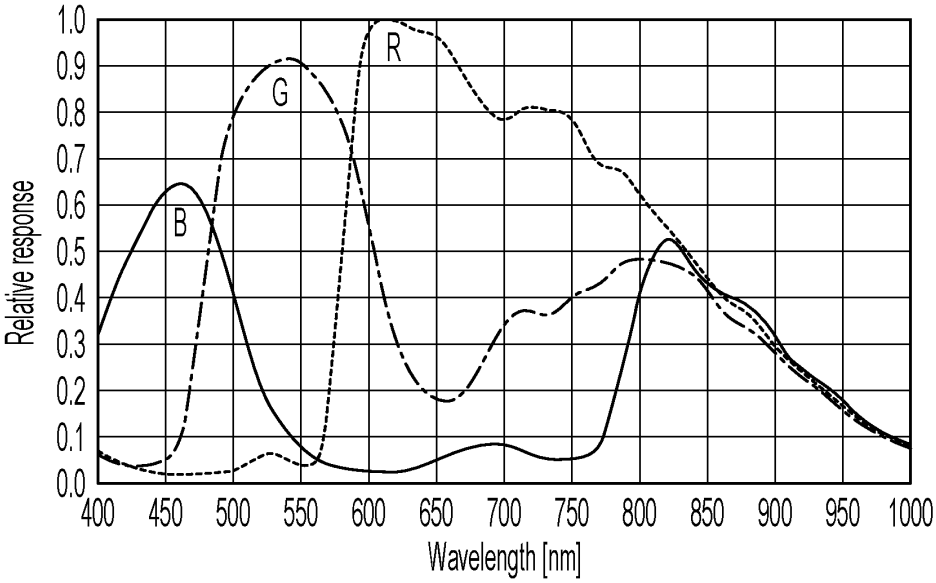

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Relative response
B
G
R
400
450
500
550
600
650
700
750
800
850
900
950
1000
Wavelength [nm]

FIG. 9

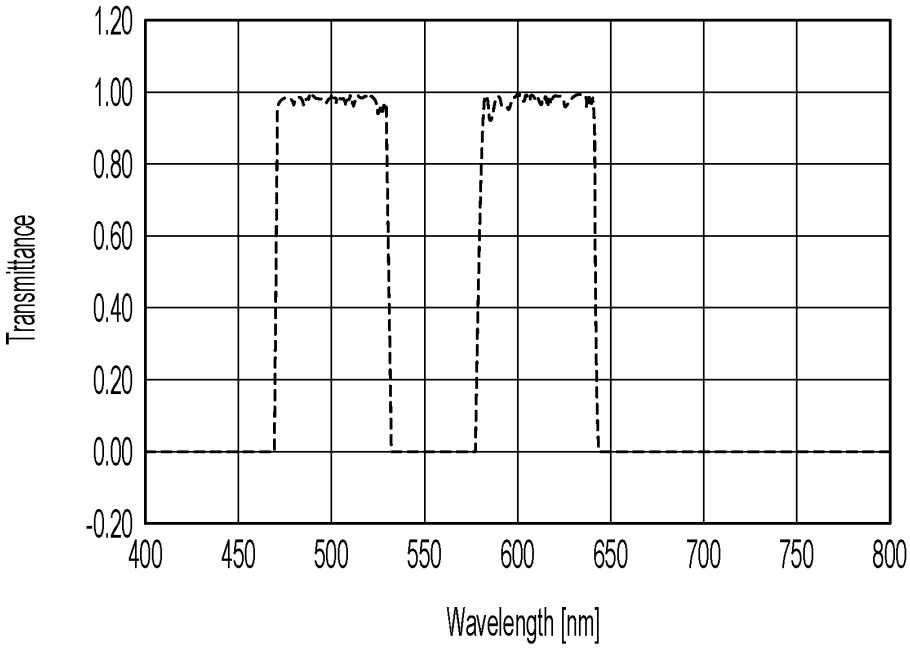

1.20
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
Transmittance
400
450
500
550
600
650
700
750
800
Wavelength [nm]

FIG. 10

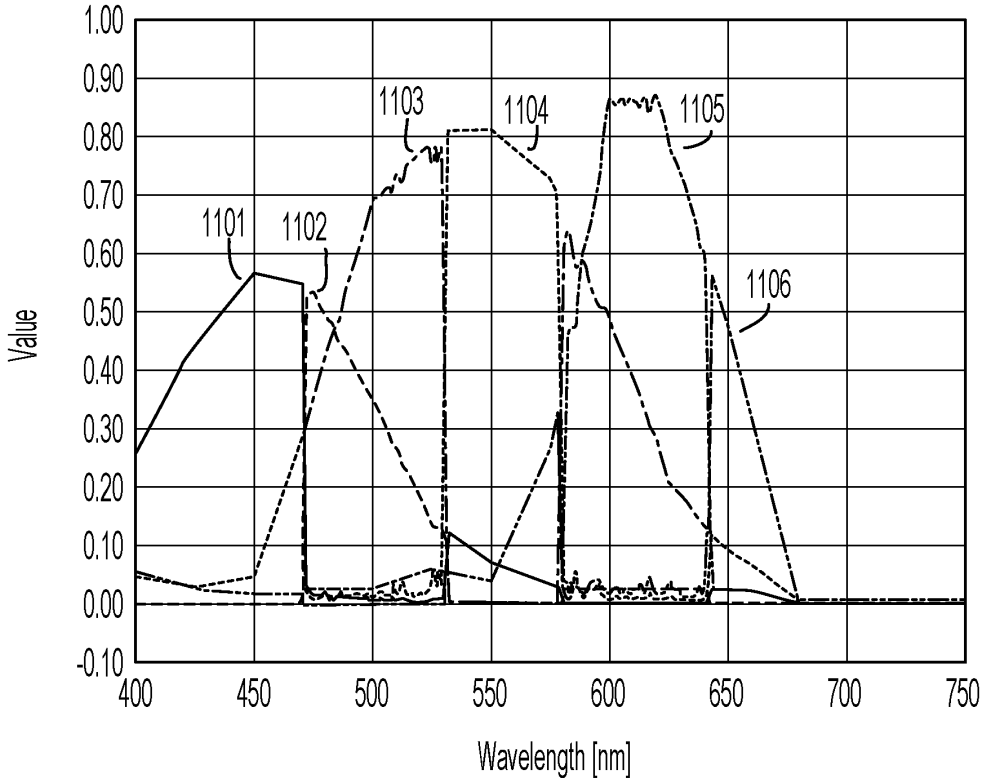

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
Value
400
450
500
550
600
650
700
750
Wavelength [nm]
1101
1102
1103
1104
1105
1106

FIG. 11

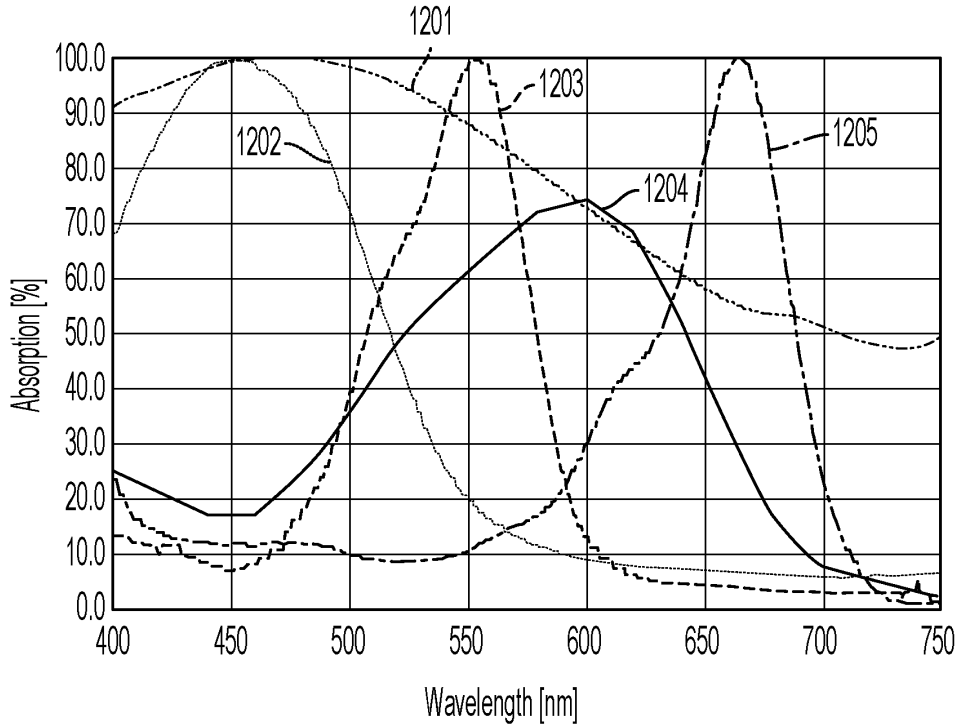

1201
1202
1203
1204
1205
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Absorption [%]
400
450
500
550
600
650
700
750
Wavelength [nm]

FIG. 12

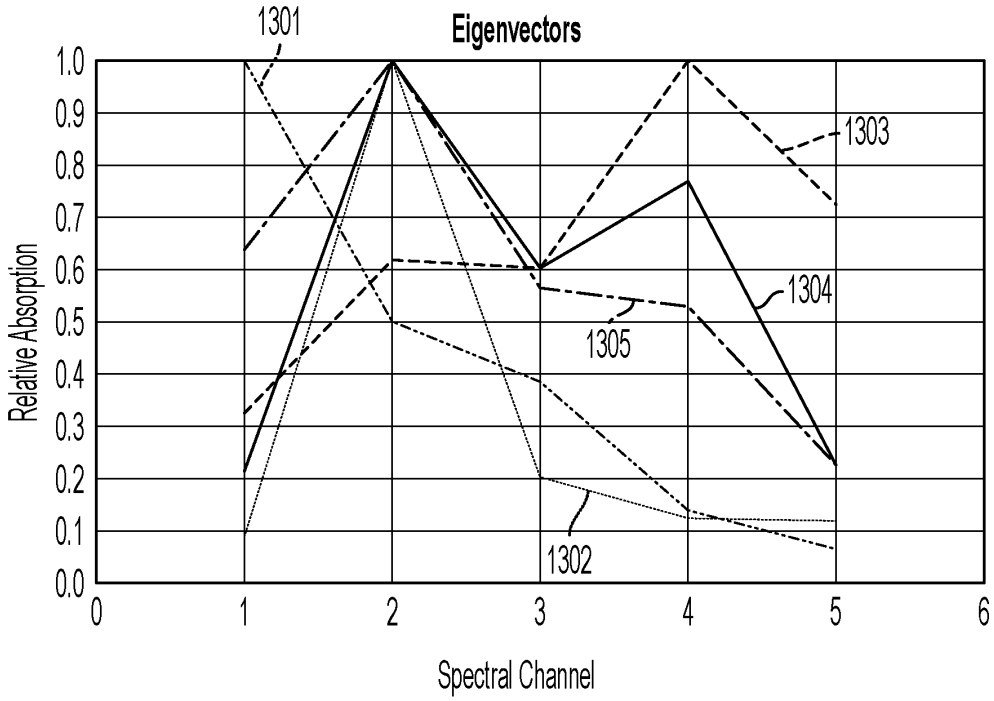

Eigenvectors
1301
1303
1304
1305
1302
Relative Absorption
Spectral Channel
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
1
2
3
4
5
6

FIG. 13

1440a

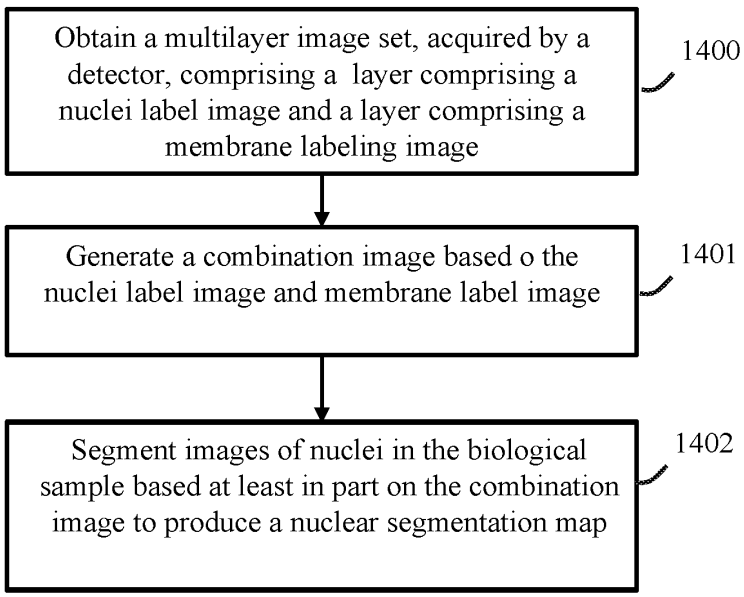


FIG. 14A

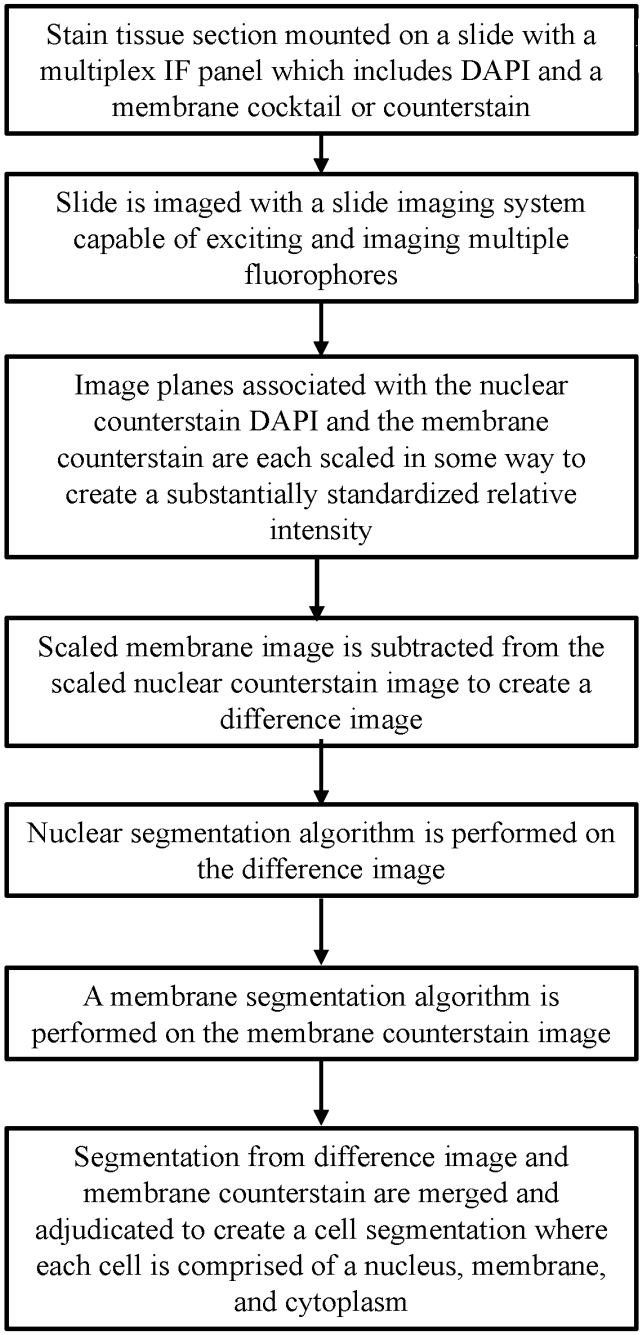

Stain tissue section mounted on a slide with a multiplex IF panel which includes DAPI and a membrane cocktail or counterstain
Slide is imaged with a slide imaging system capable of exciting and imaging multiple fluorophores
Image planes associated with the nuclear counterstain DAPI and the membrane counterstain are each scaled in some way to create a substantially standardized relative intensity
Scaled membrane image is subtracted from the scaled nuclear counterstain image to create a difference image
Nuclear segmentation algorithm is performed on the difference image
A membrane segmentation algorithm is performed on the membrane counterstain image
Segmentation from difference image and membrane counterstain are merged and adjudicated to create a cell segmentation where each cell is comprised of a nucleus, membrane, and cytoplasm

FIG. 14B

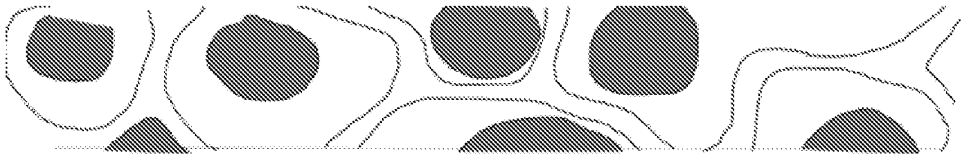


FIG. 15

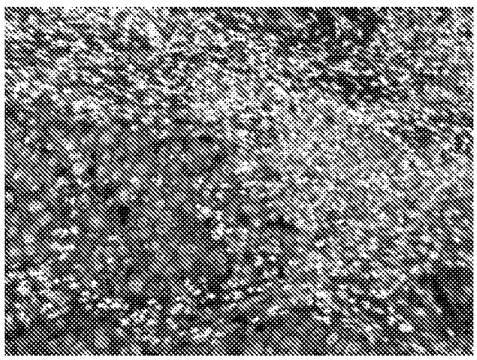


FIG. 16

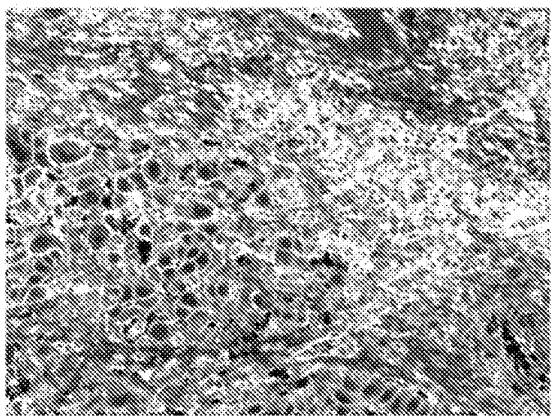


FIG. 17

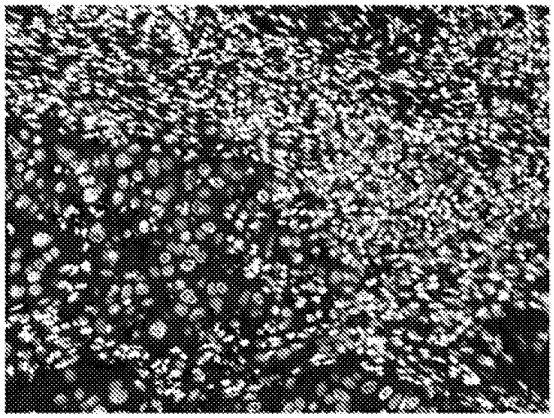


FIG. 18

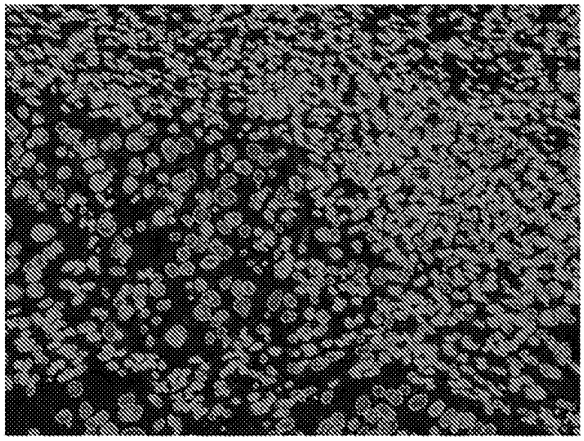


FIG. 19

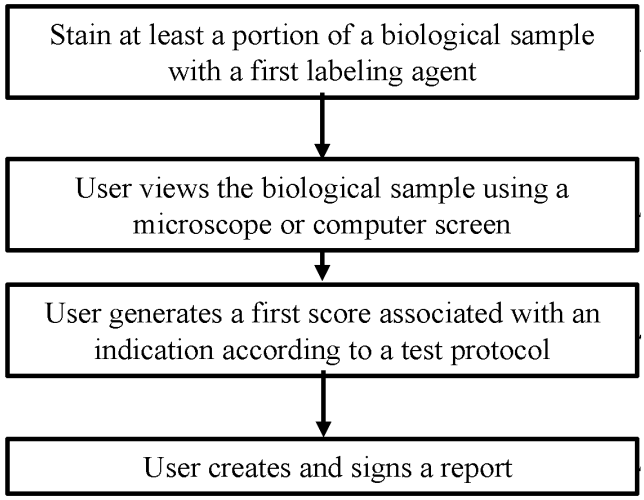

Stain at least a portion of a biological sample with a first labeling agent
User views the biological sample using a microscope or computer screen
User generates a first score associated with an indication according to a test protocol
User creates and signs a report

FIG. 20

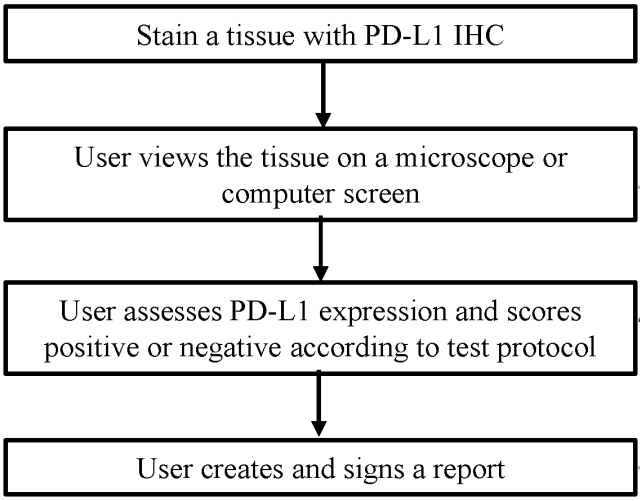

Stain a tissue with PD-L1 IHC
User views the tissue on a microscope or computer screen
User assesses PD-L1 expression and scores positive or negative according to test protocol
User creates and signs a report

FIG. 21

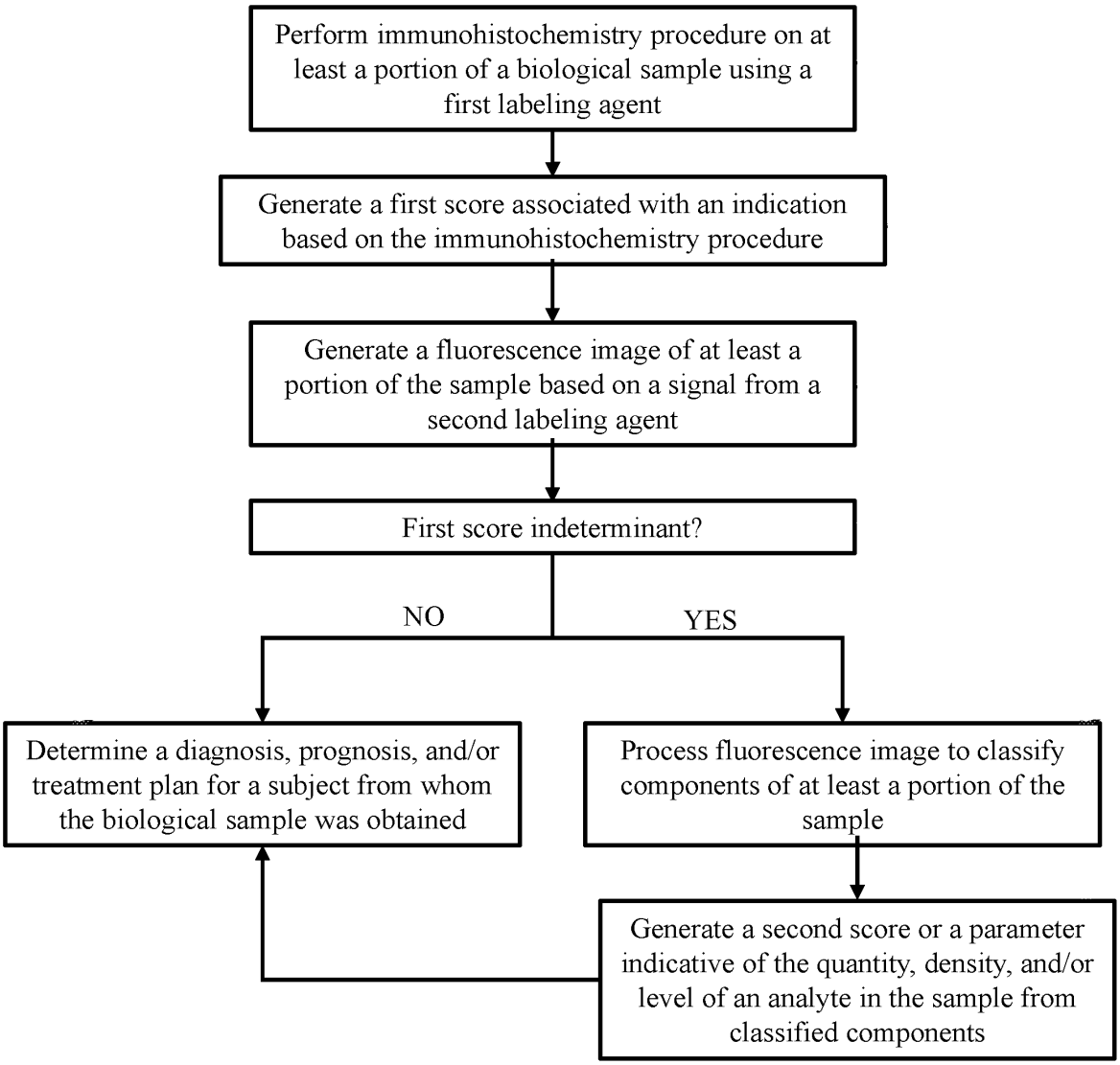

Perform immunohistochemistry procedure on at least a portion of a biological sample using a first labeling agent
Generate a first score associated with an indication based on the immunohistochemistry procedure
Generate a fluorescence image of at least a portion of the sample based on a signal from a second labeling agent
First score indeterminant?
NO
YES
Determine a diagnosis, prognosis, and/or treatment plan for a subject from whom the biological sample was obtained
Process fluorescence image to classify components of at least a portion of the sample
Generate a second score or a parameter indicative of the quantity, density, and/or level of an analyte in the sample from classified components

FIG. 22

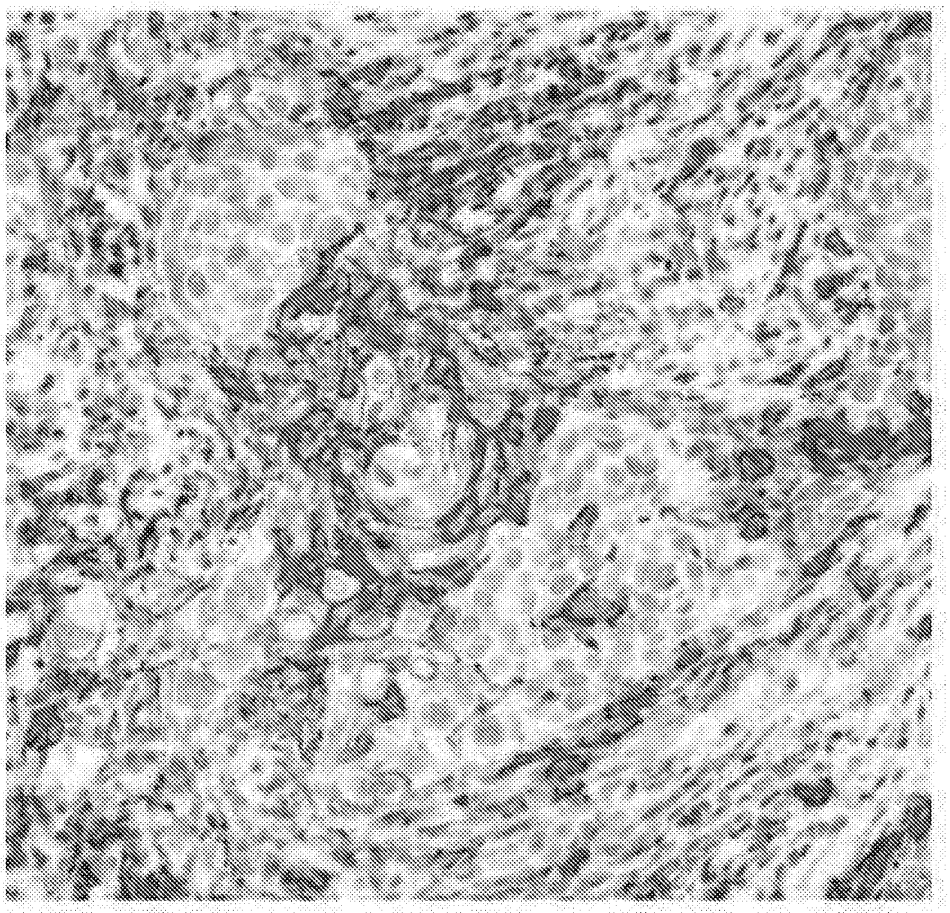


FIG. 23

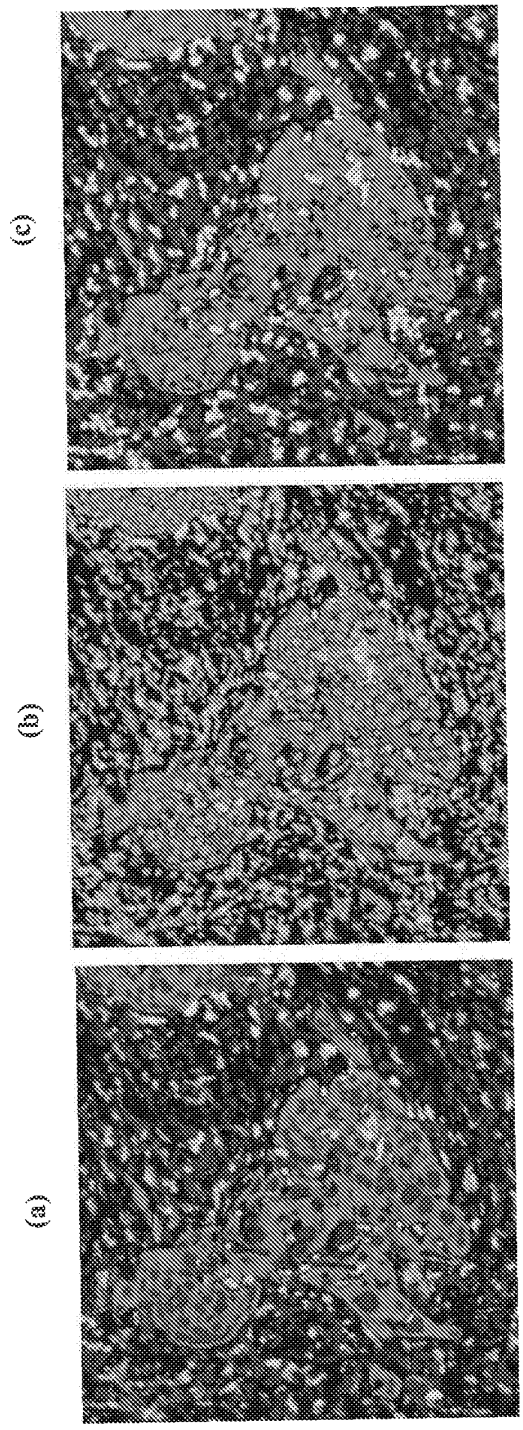


FIG. 24

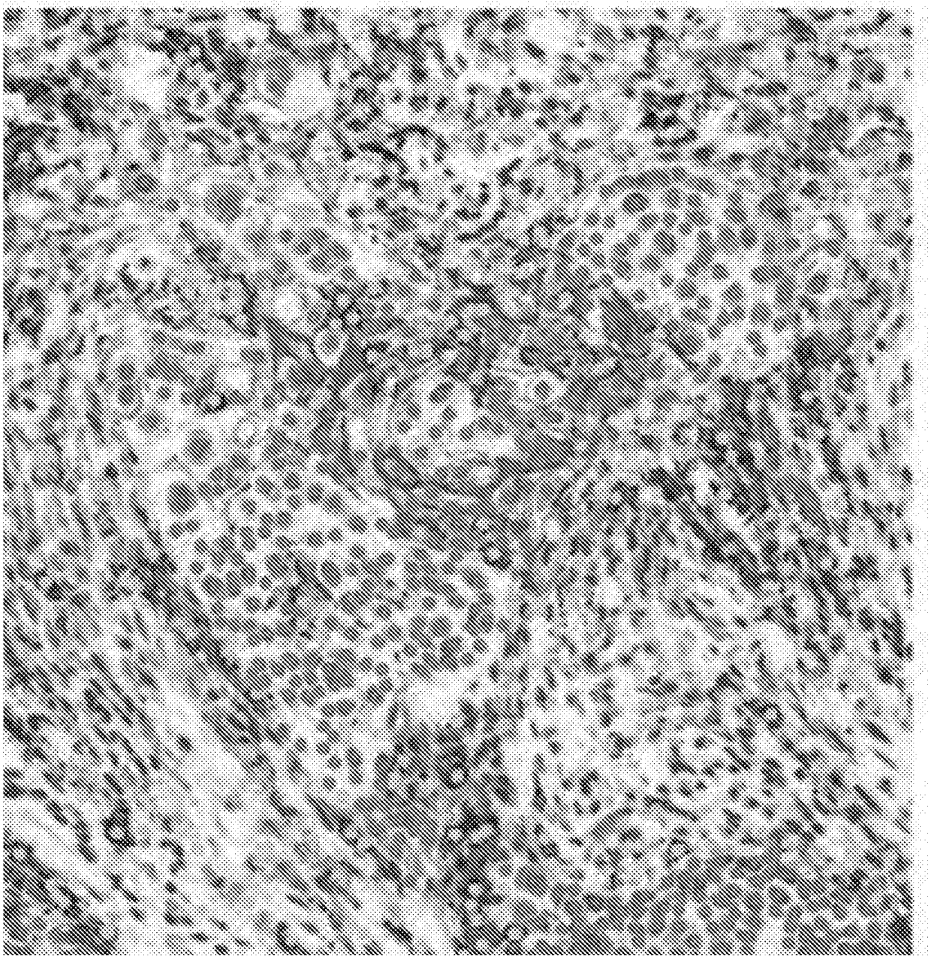


FIG. 25

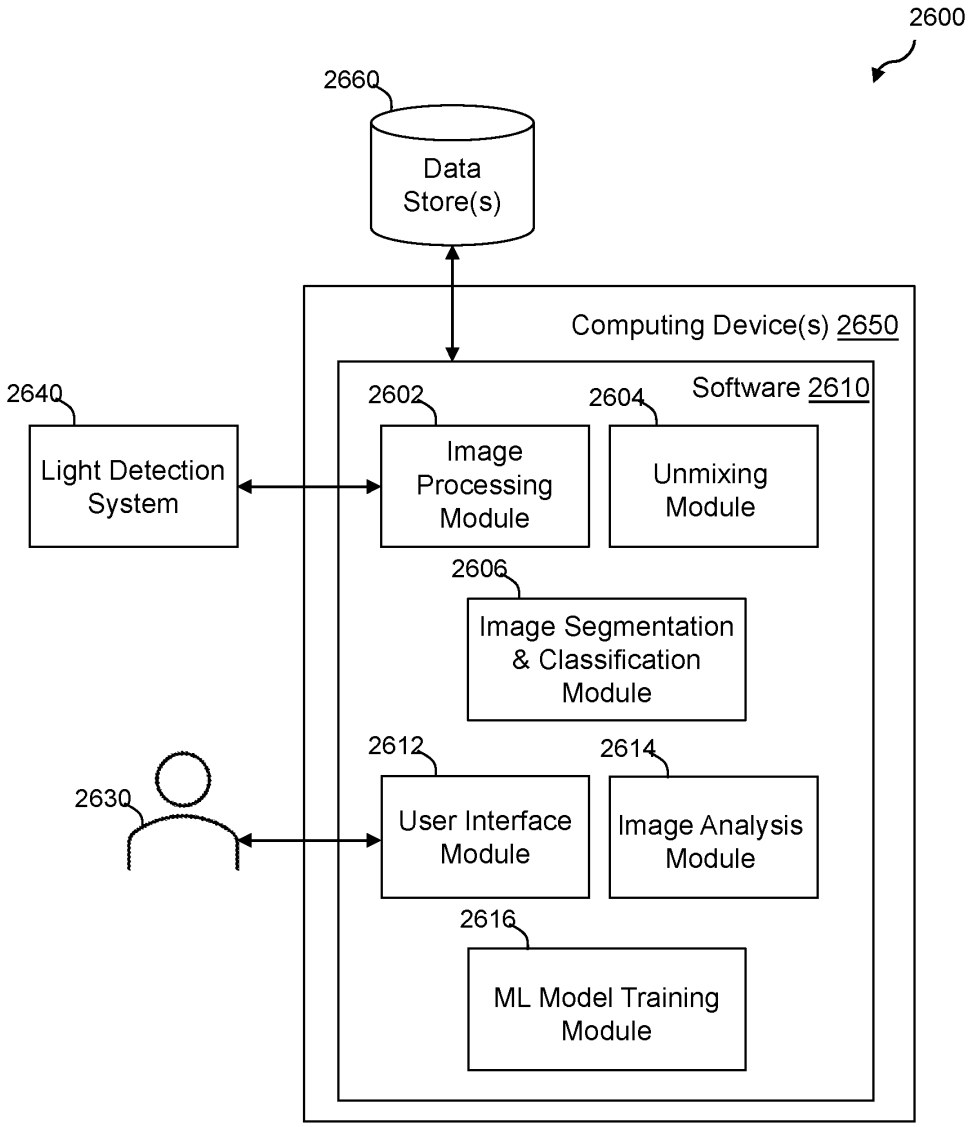

2600
2660
Data Store(s)
Computing Device(s) 2650
Software 2610
2640
Light Detection System
2602
Image Processing Module
2604
Unmixing Module
2606
Image Segmentation & Classification Module
2630
2612
User Interface Module
2614
Image Analysis Module
2616
ML Model Training Module

FIG. 26

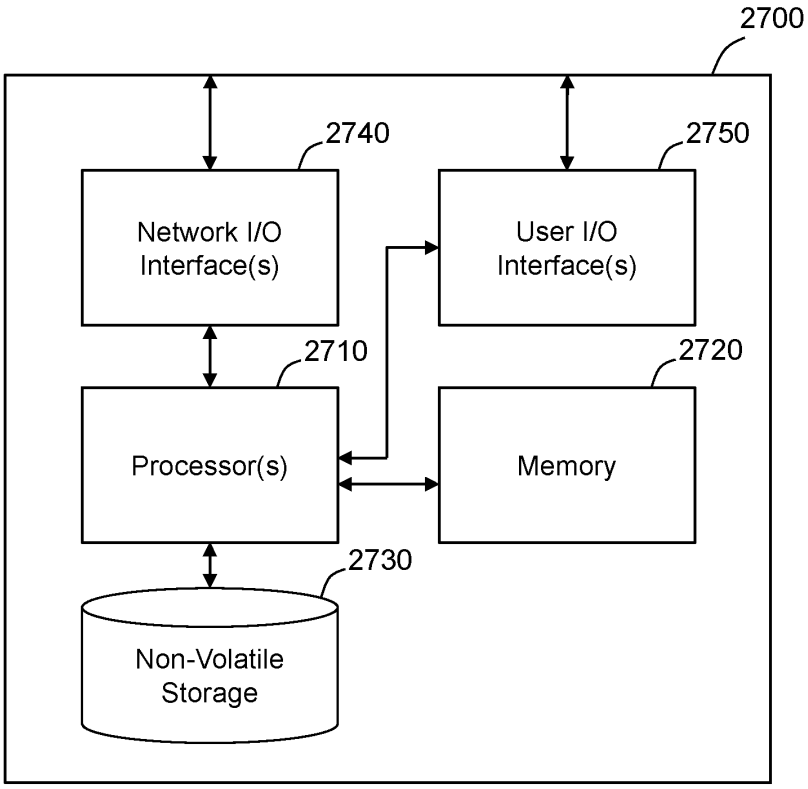

2700
2740
2750
Network I/O Interface(s)
User I/O Interface(s)
2710
2720
Processor(s)
Memory
2730
Non-Volatile Storage

FIG. 27

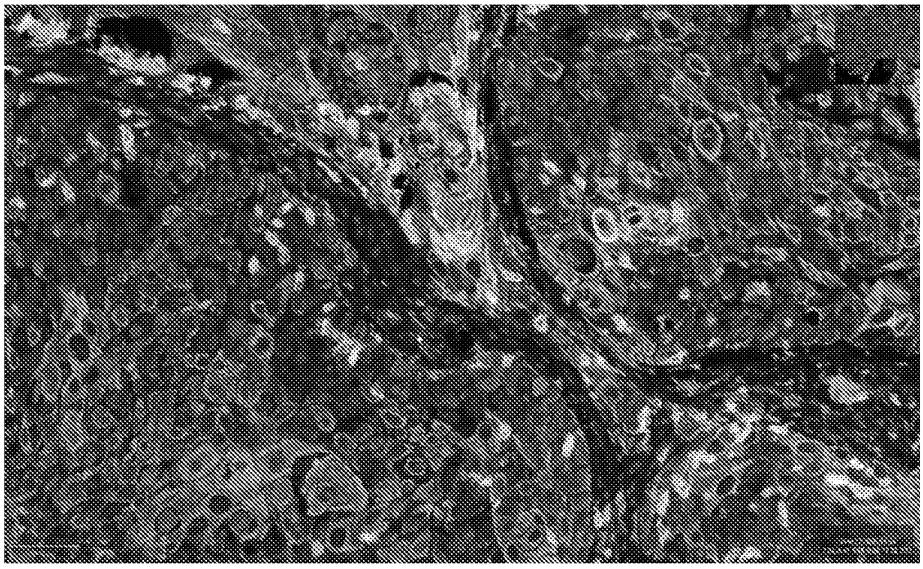


FIG. 28

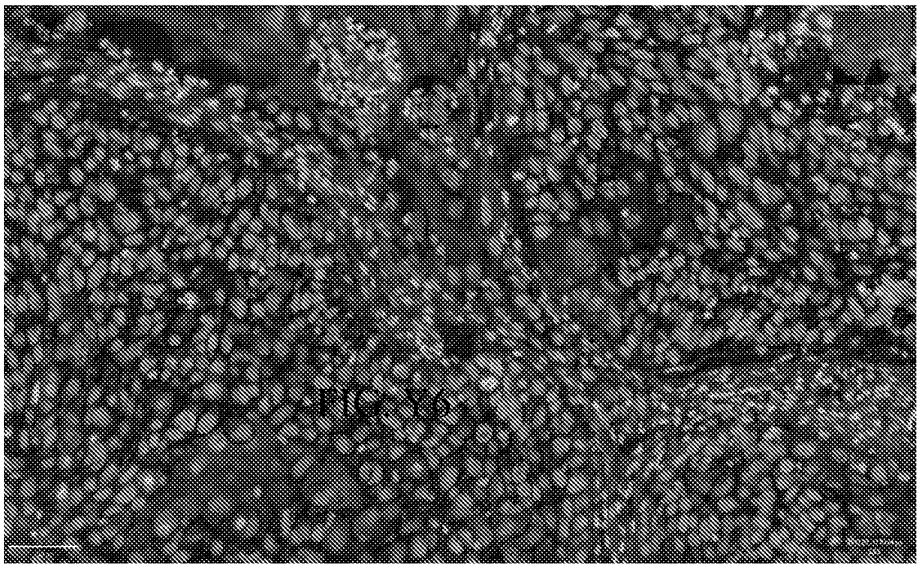


FIG. 29

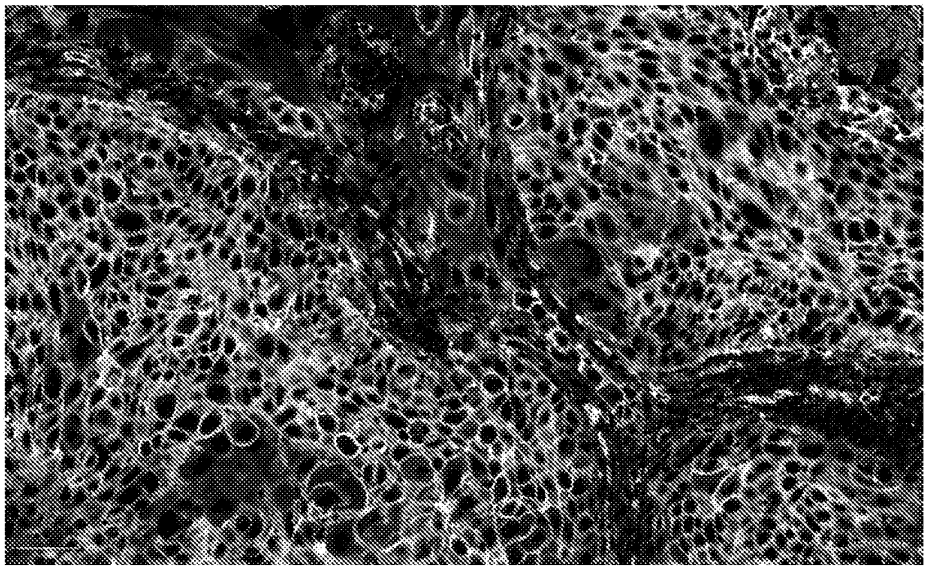


FIG. 30

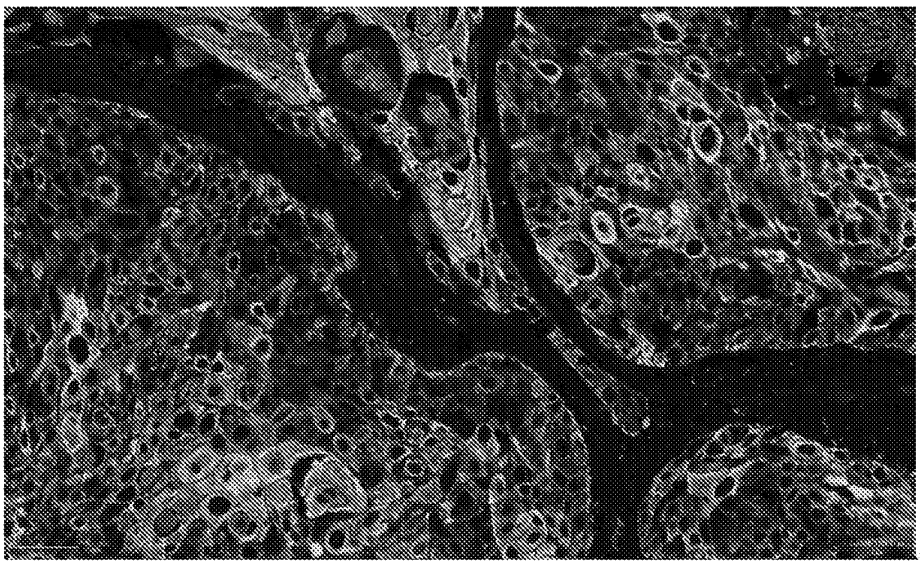


FIG. 31

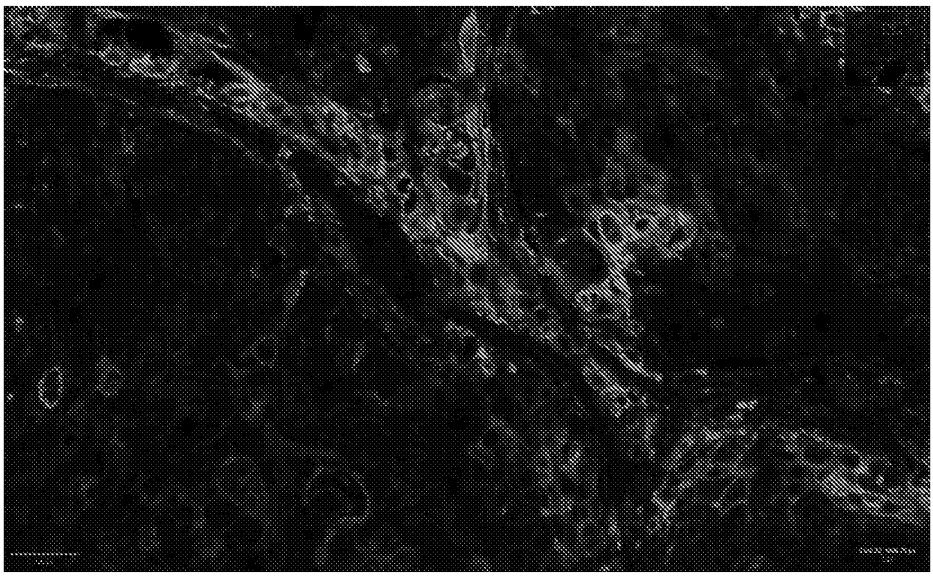


FIG. 32

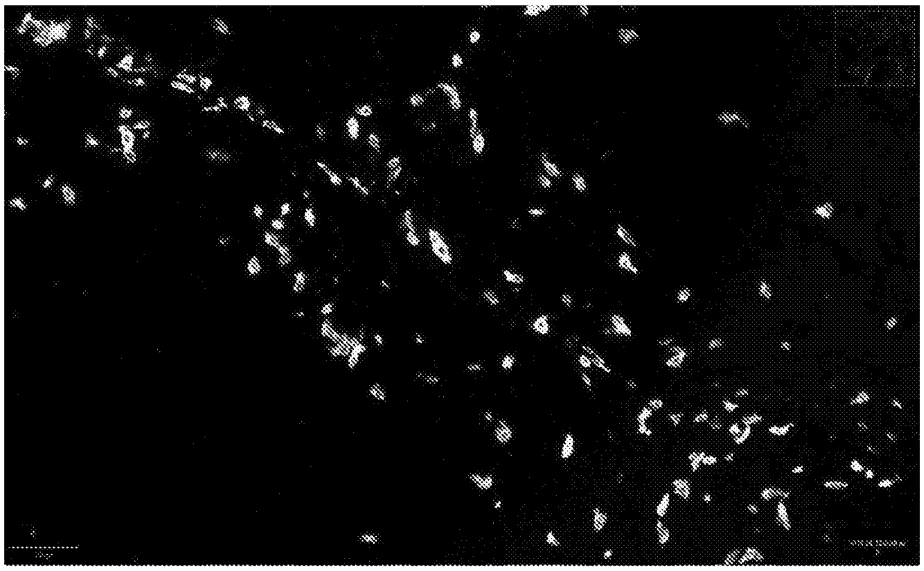


FIG. 33

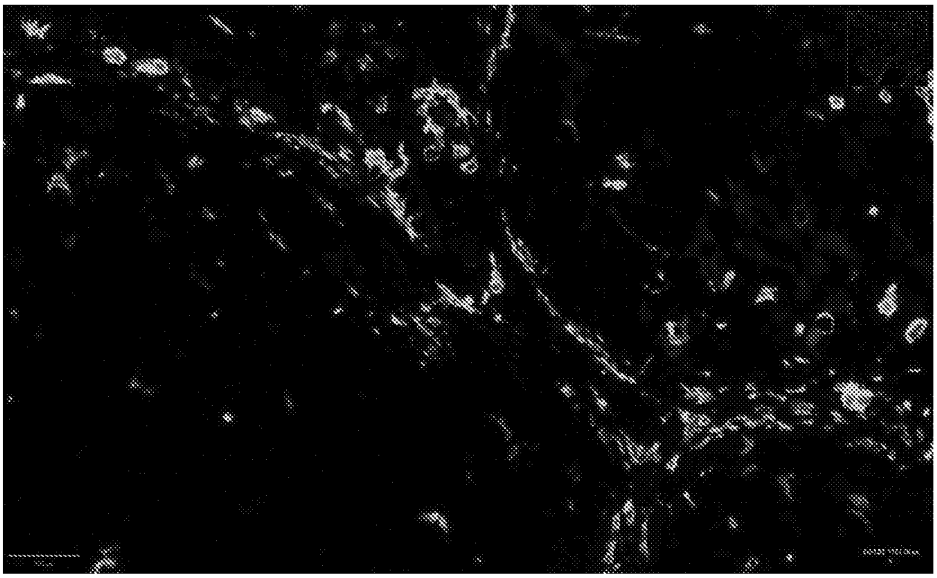


FIG. 34