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(54) **QUALITY EVALUATION METHOD FOR SPHEROID INCLUDING HEPATOCYTES**

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(57) **ABSTRACT**

In an evaluation method, first, spheroids obtained by three-dimensional culture of multiple kinds of liver-derived cells are imaged by optical coherence photography, a localization region is extracted from the photographic image, the localization region is analyzed (analysis step), and the condition of the spheroid is evaluated. The analysis step includes a first calculation step of calculating the area of the entire spheroid in the photographic image, a second calculation step of calculating the area of the localization region, a third calculation step of calculating the ratio of the localization region on the basis of the two areas, and a fourth calculation step of calculating an evaluation parameter on the basis of the ratio.

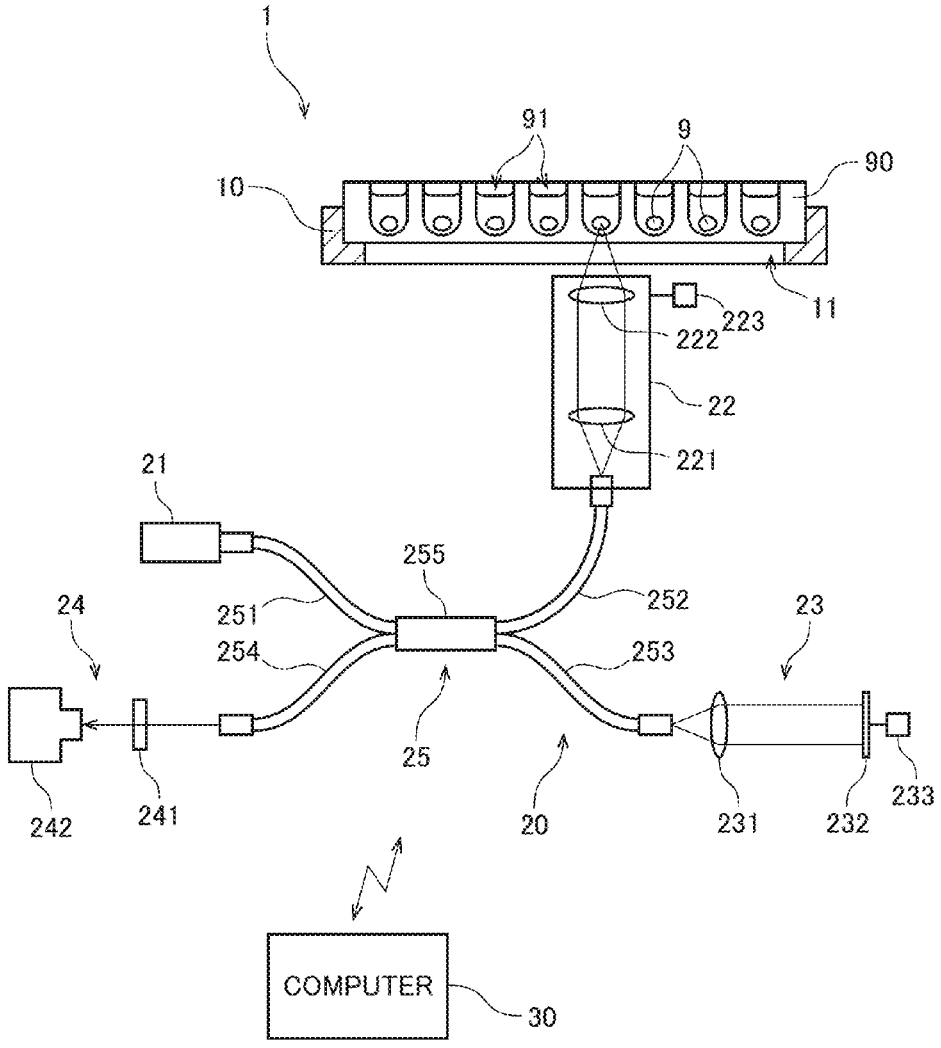


FIG. 1

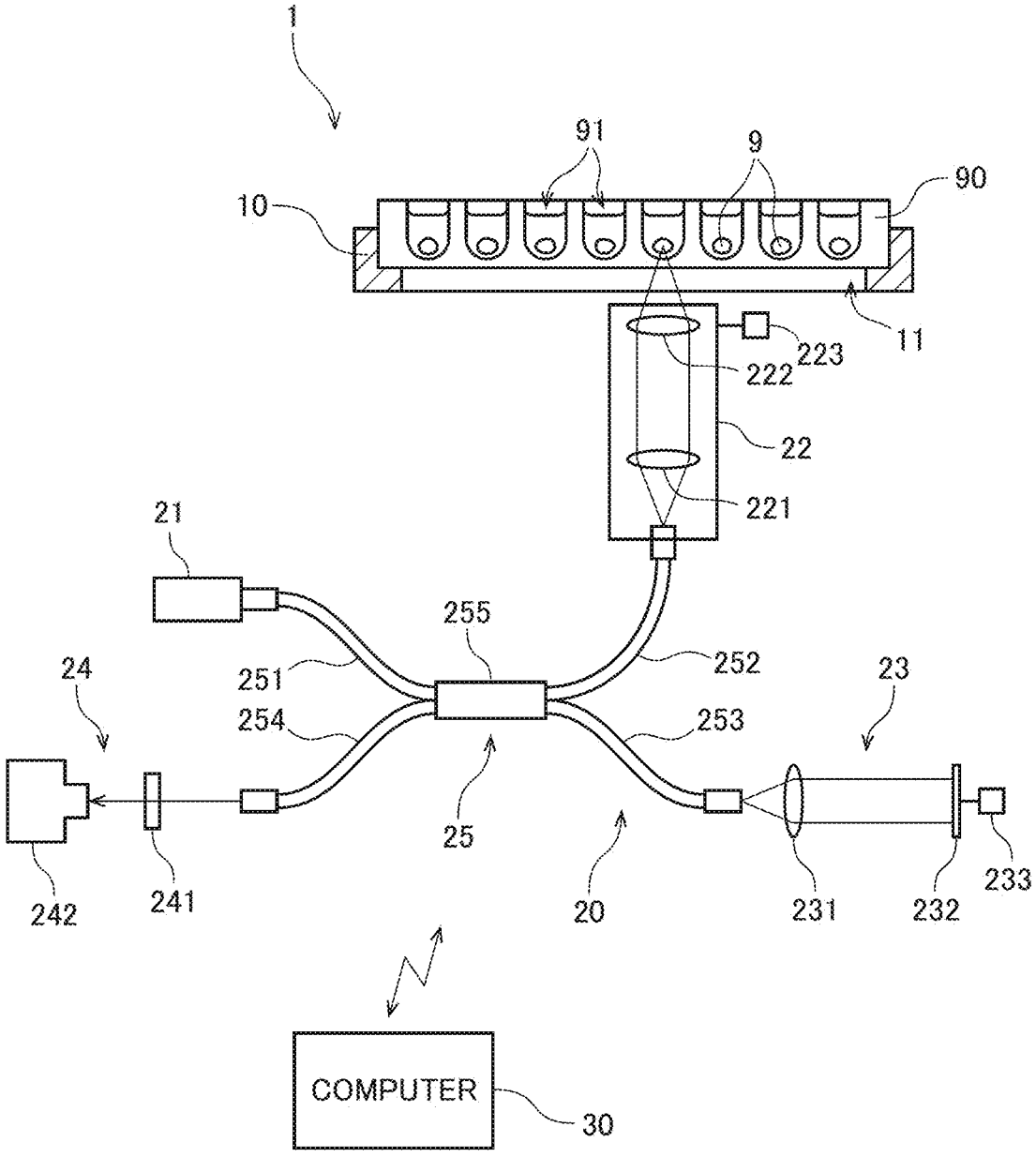

1
91
9
90
10
11
223
222
22
221
21
255
24
251
252
23
254
253
25
20
242
241
231
232
233
COMPUTER
30

FIG. 2

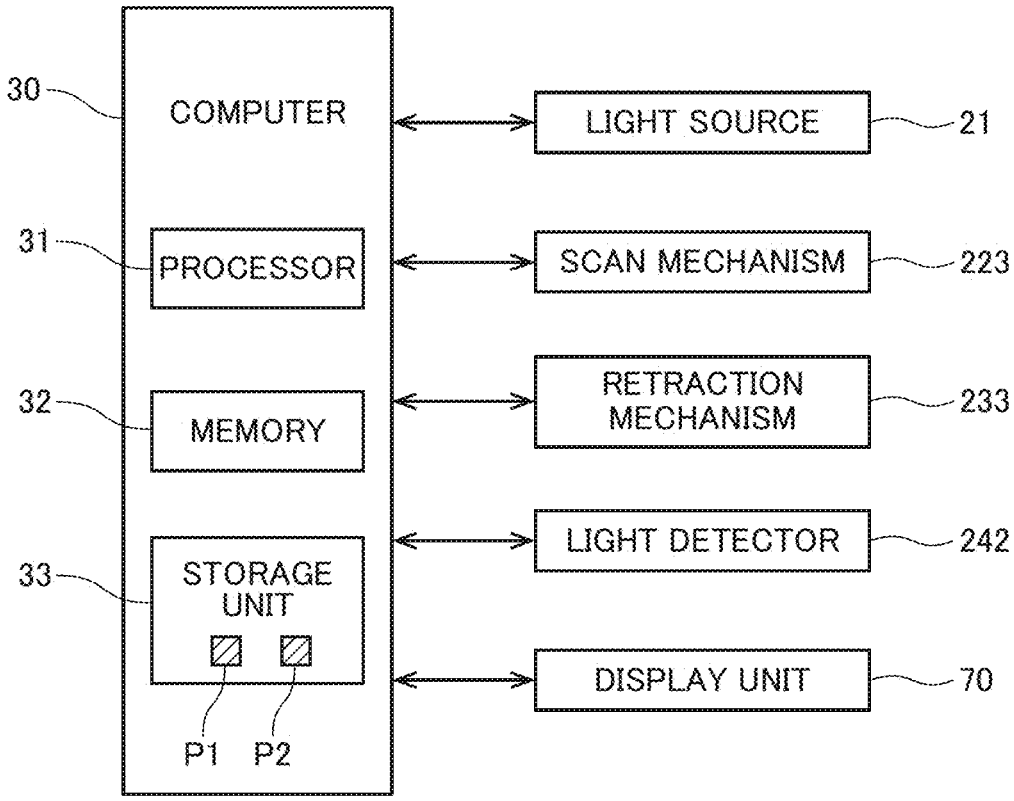

30
COMPUTER
31
PROCESSOR
32
MEMORY
33
STORAGE UNIT
P1
P2
LIGHT SOURCE
21
SCAN MECHANISM
223
RETRACTION MECHANISM
233
LIGHT DETECTOR
242
DISPLAY UNIT
70

FIG. 3

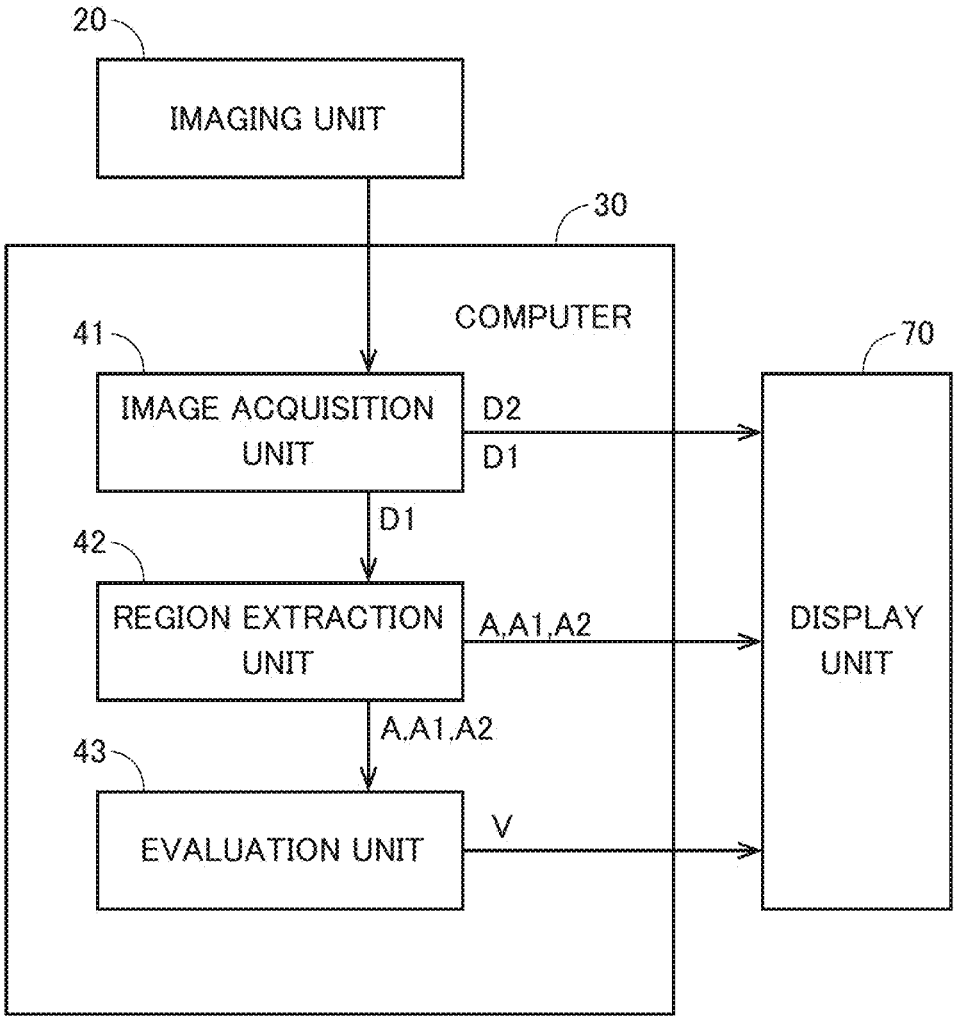

20
IMAGING UNIT
30
COMPUTER
41
IMAGE ACQUISITION UNIT
D2
D1
70
D1
42
REGION EXTRACTION UNIT
A,A1,A2
DISPLAY UNIT
A,A1,A2
43
EVALUATION UNIT
V

FIG. 4A

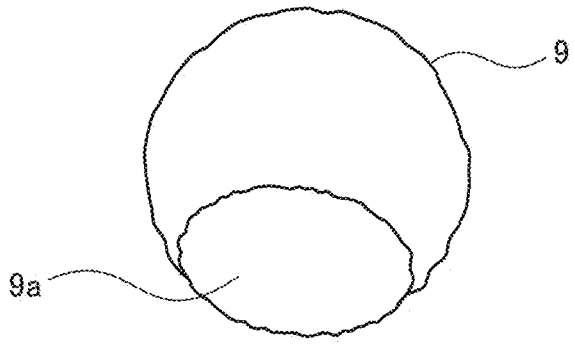


FIG. 4B

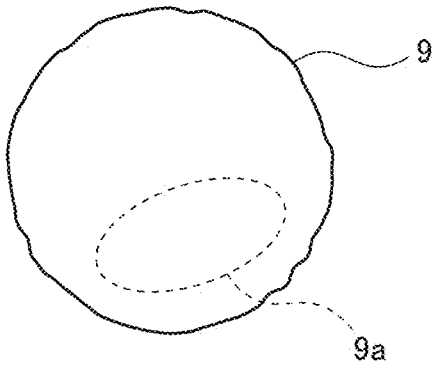


FIG. 5

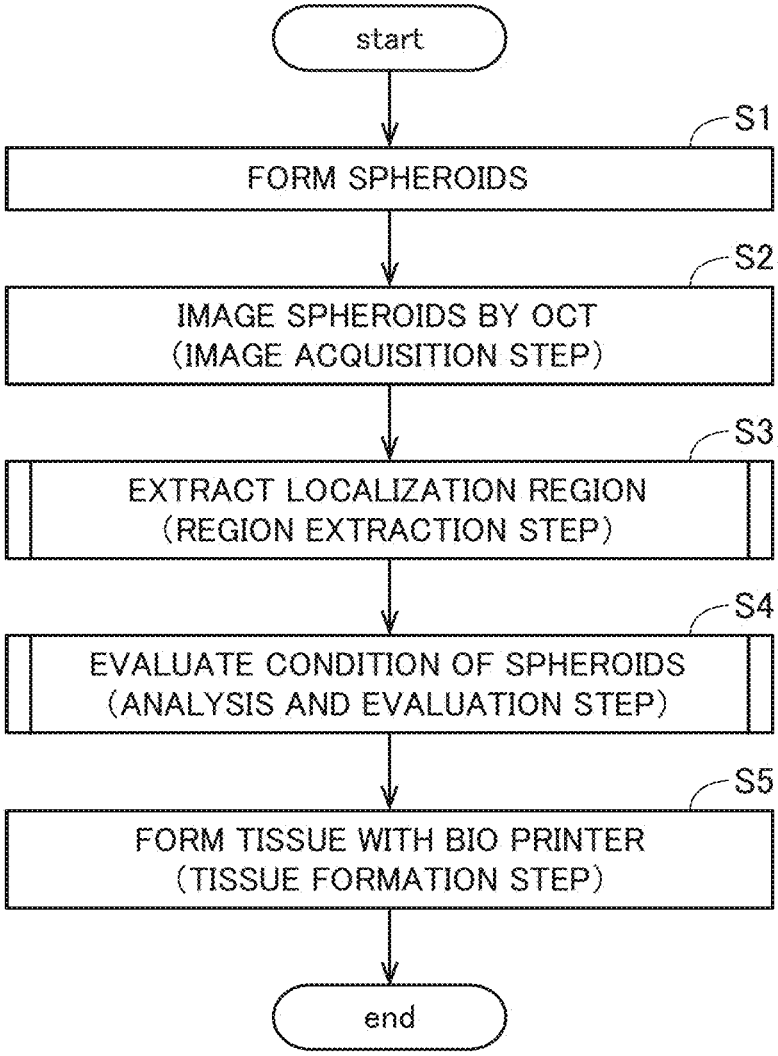

start
S1
FORM SPHEROIDS
S2
IMAGE SPHEROIDS BY OCT
(IMAGE ACQUISITION STEP)
S3
EXTRACT LOCALIZATION REGION
(REGION EXTRACTION STEP)
S4
EVALUATE CONDITION OF SPHEROIDS
(ANALYSIS AND EVALUATION STEP)
S5
FORM TISSUE WITH BIO PRINTER
(TISSUE FORMATION STEP)
end

FIG. 6

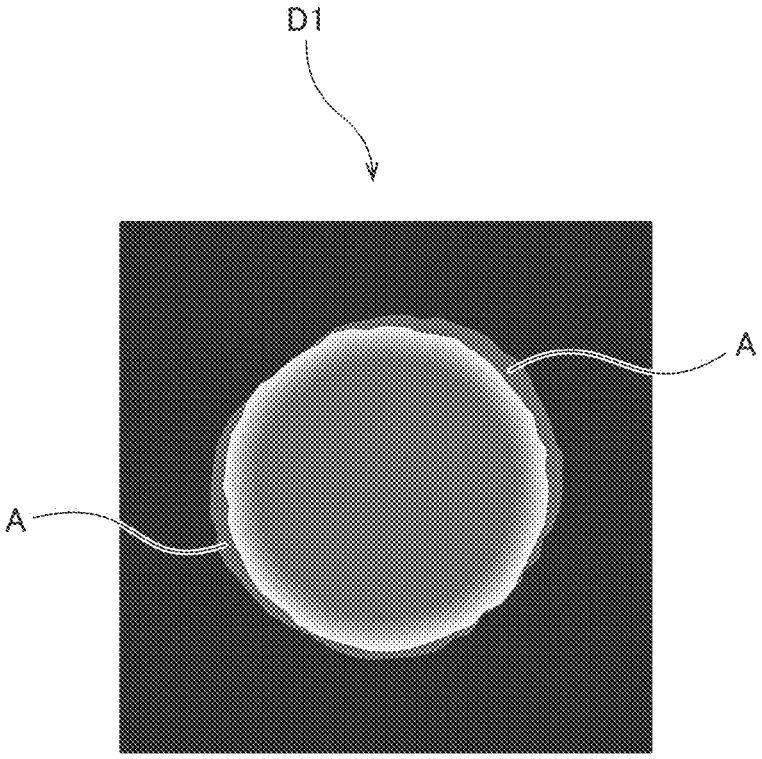


FIG. 7

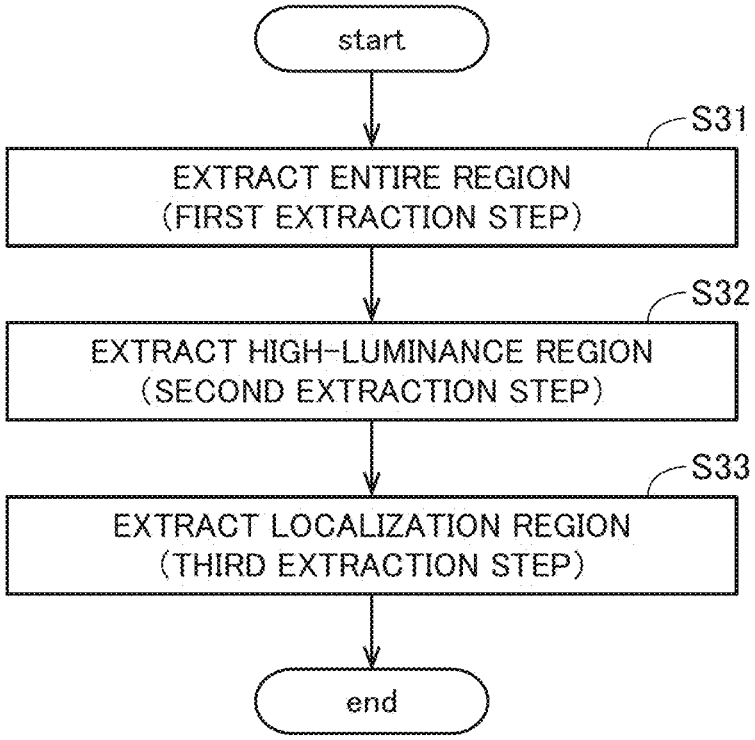

start
S31
EXTRACT ENTIRE REGION
(FIRST EXTRACTION STEP)
S32
EXTRACT HIGH-LUMINANCE REGION
(SECOND EXTRACTION STEP)
S33
EXTRACT LOCALIZATION REGION
(THIRD EXTRACTION STEP)
end

FIG. 8

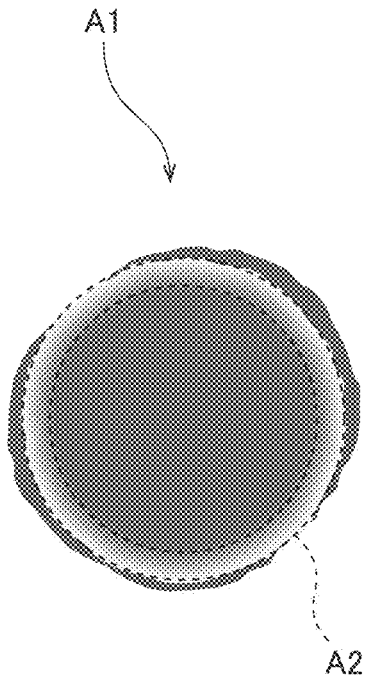


FIG. 9

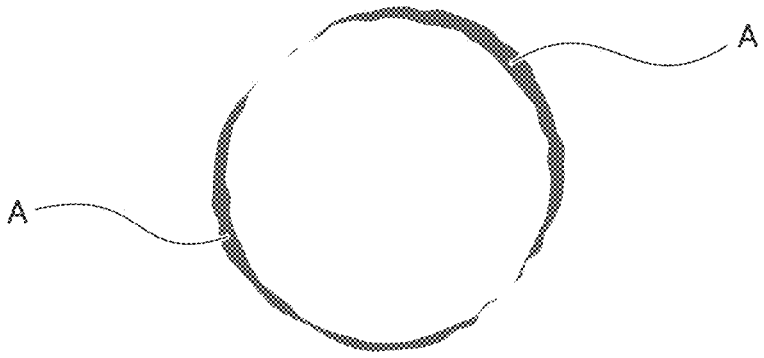


FIG. 10

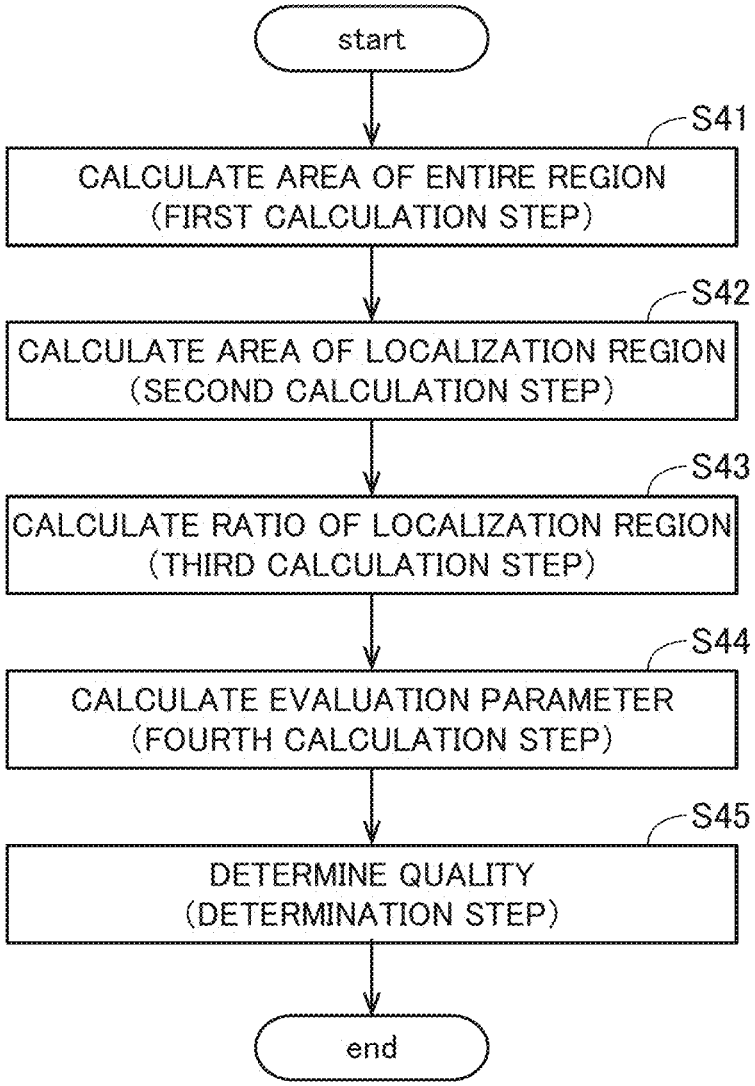

start
S41
CALCULATE AREA OF ENTIRE REGION
(FIRST CALCULATION STEP)
S42
CALCULATE AREA OF LOCALIZATION REGION
(SECOND CALCULATION STEP)
S43
CALCULATE RATIO OF LOCALIZATION REGION
(THIRD CALCULATION STEP)
S44
CALCULATE EVALUATION PARAMETER
(FOURTH CALCULATION STEP)
S45
DETERMINE QUALITY
(DETERMINATION STEP)
end

FIG. 11

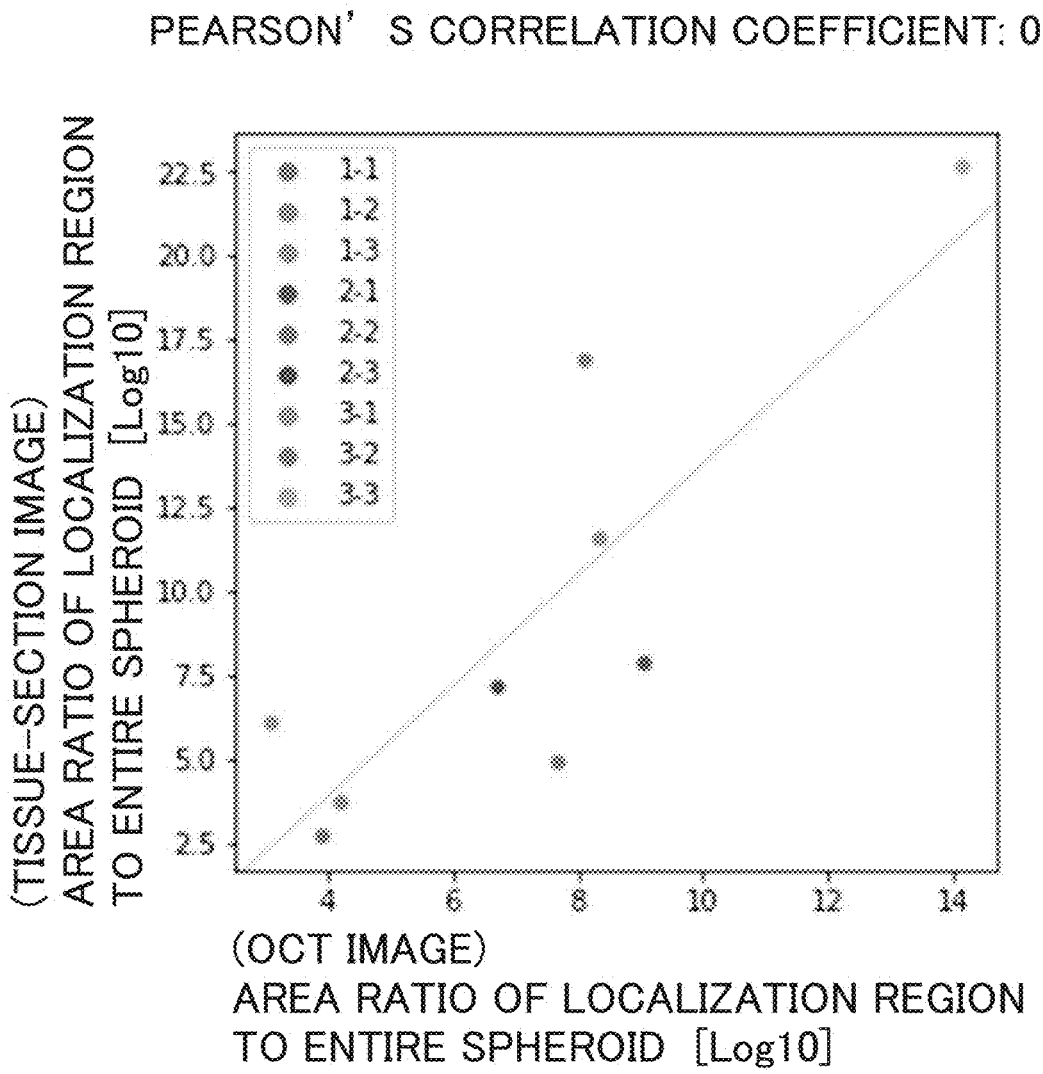

PEARSON' S CORRELATION COEFFICIENT: 0.839
(TISSUE-SECTION IMAGE)
AREA RATIO OF LOCALIZATION REGION
TO ENTIRE SPHEROID [Log10]
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
1-1
1-2
1-3
2-1
2-2
2-3
3-1
3-2
3-3
4
6
8
10
12
14
(OCT IMAGE)
AREA RATIO OF LOCALIZATION REGION
TO ENTIRE SPHEROID [Log10]

FIG. 12

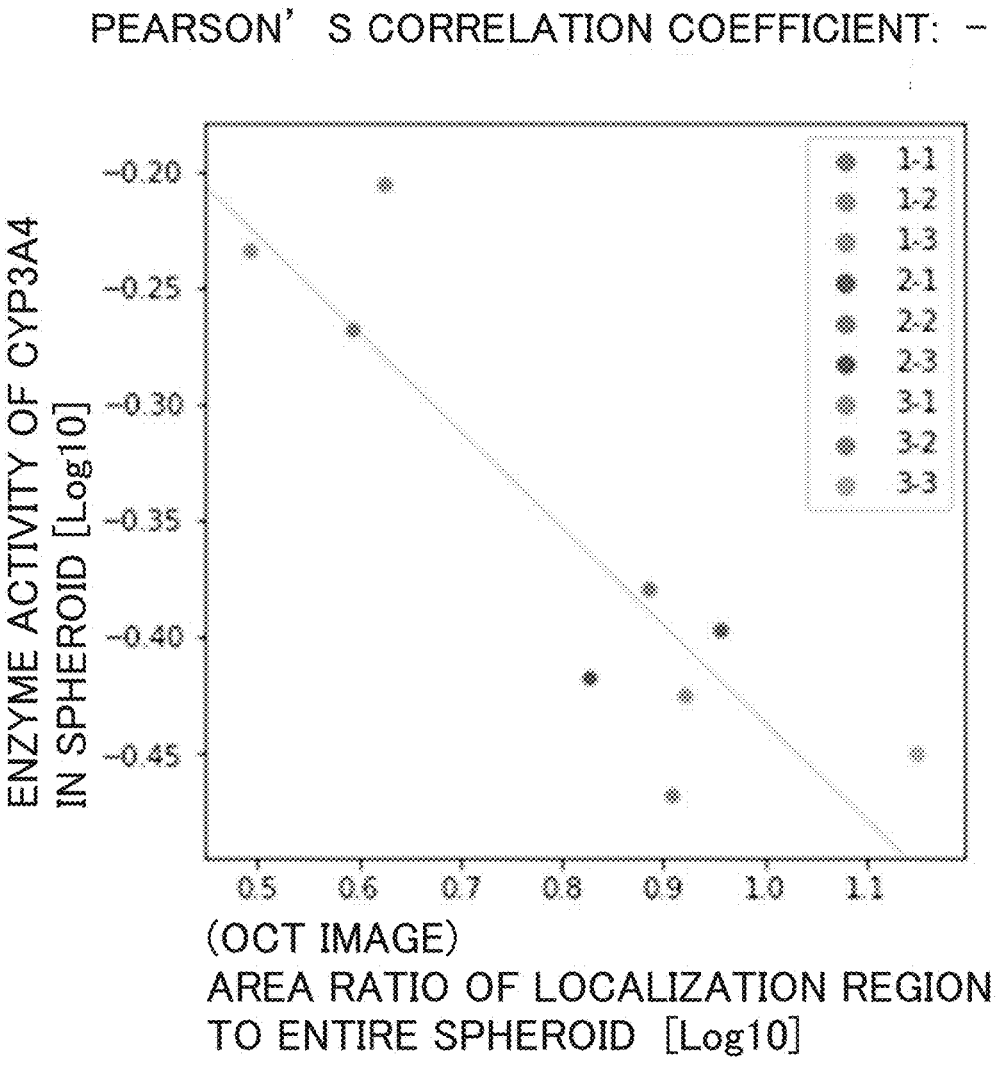
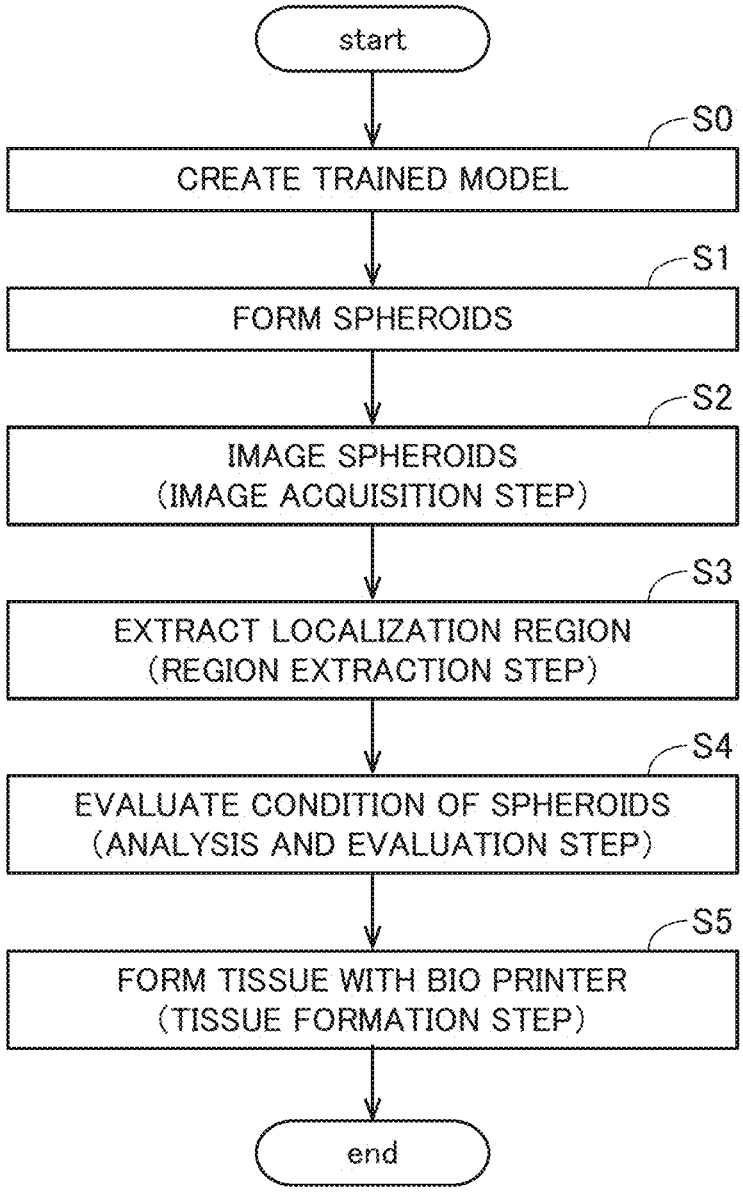

PEARSON' S CORRELATION COEFFICIENT: − 0.885
ENZYME ACTIVITY OF CYP3A4 IN SPHEROID [Log10]
−0.20
−0.25
−0.30
−0.35
−0.40
−0.45
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1-1
1-2
1-3
2-1
2-2
2-3
3-1
3-2
3-3
(OCT IMAGE)
AREA RATIO OF LOCALIZATION REGION TO ENTIRE SPHEROID [Log10]

FIG. 13


start
S0
CREATE TRAINED MODEL
S1
FORM SPHEROIDS
S2
IMAGE SPHEROIDS
(IMAGE ACQUISITION STEP)
S3
EXTRACT LOCALIZATION REGION
(REGION EXTRACTION STEP)
S4
EVALUATE CONDITION OF SPHEROIDS
(ANALYSIS AND EVALUATION STEP)
S5
FORM TISSUE WITH BIO PRINTER
(TISSUE FORMATION STEP)
end

QUALITY EVALUATION METHOD FOR SPHEROID INCLUDING HEPATOCYTES

RELATED APPLICATIONS

[0001] This application claims the benefit of Japanese Application No. 2023-037283, filed on Mar. 10, 2023, the disclosure of which is incorporated by reference herein.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention relates to an evaluation method for spheroids.

Description of the Background Art

[0003] There is conventionally known an observation apparatus that images a biological specimen consisting of a plurality of cells by optical coherence tomography (OCT), and observes the biological sample on the basis of the obtained tomographic image. Such a conventional observation apparatus as mentioned above is described in, for example, Japanese Patent Application Publication No. 2015-181348. With the use of this type of observation apparatus, it is possible to noninvasively observe a three-dimensional structure of a biological sample.

[0004] A biological sample such as a spheroid includes an unusual part in which the status of cells is different from that in the other parts, in some cases. For example, in a case in which multiple kinds of cells are combined to form a biological sample such as a spheroid, certain kinds of cells may probably be localized on the outside or on the inside of the biological sample. Further, in a biological sample such as a spheroid that includes stem cells and the other kinds of cells, differentiated cells may probably be localized in a part of the biological sample. In quality control of the biological sample, it is very important to observe those unusual parts.

[0005] As a conventional method for observing an unusual part such as a localization region of a spheroid, a method of staining a section to be observed with the use of a fluorescent reagent is known. However, this method causes damages to an object to be observed. Hence, there is a demand for a technology for observing an unusual part of a biological sample in a natural condition as it is without causing damages to the unusual part.

SUMMARY OF THE INVENTION

[0006] The present invention has been made in view of the above-described situation, and it is an object to provide an evaluation method that makes it possible to noninvasively evaluate the condition of a spheroid.

[0007] To solve the above-described problem, the first invention of the present application is directed to an evaluation method for a spheroid. The spheroid is a spheroid including multiple kinds of liver-derived cells including a hepatocyte, and the evaluation method includes: an image acquisition step of imaging the spheroid by optical coherence tomography and acquiring a photographic image; a region extraction step of extracting a localization region in which one kind of cell is localized, from the photographic image; an analysis step of analyzing the localization region; and an evaluation step of evaluating a condition of the spheroid, wherein the analysis step includes: a first calculation step of calculating an area of the entire spheroid in the photographic image; a second calculation step of calculating an area of the localization region in the photographic image; a third calculation step of calculating a ratio of the localization region on the basis of the area of the entire spheroid and the area of the localization region; and a fourth calculation step of calculating an evaluation parameter on the basis of the ratio of the localization region.

[0008] The second invention of the present application is directed to the evaluation method of the first invention, wherein the evaluation step includes a determination step of determining a quality of the spheroid on the basis of the evaluation parameter.

[0009] The third invention of the present application is directed to the evaluation method of the first invention, wherein the evaluation parameter is an estimated value of a secretion amount of predetermined protein in the spheroid.

[0010] The fourth invention of the present application is directed to the evaluation method of the third invention, wherein the evaluation parameter is an estimated value of enzyme activity of CYP3A4 in the spheroid.

[0011] The fifth invention of the present application is directed to the evaluation method of any of the first to fourth inventions, wherein the region extraction step includes: a first extraction step of extracting an entire region corresponding to the spheroid, from the photographic image; a second extraction step of extracting a high-intensity region of which intensity value is higher than a predetermined threshold value in the entire region; and a third extraction step of extracting a region that is left after the high-intensity region and a region inner than the high-intensity region are excluded from the entire region, as the localization region.

[0012] The sixth invention of the present application is directed to an evaluation method for a spheroid. The spheroid is a spheroid including multiple kinds of liver-derived cells including a hepatocyte, and the evaluation method includes: a training step of creating a trained model configured to receive a photographic image of the spheroid imaged by optical coherence tomography, as input information, and produce a localization region in which one kind of cell is localized, as output information, by deep learning; an image acquisition step of imaging the spheroid by optical coherence tomography and acquiring a photographic image; a region extraction step of inputting the photographic image to the trained model, to extract the localization region output from the trained model; an analysis step of analyzing the localization region; and an evaluation step of evaluating a condition of the spheroid, wherein the analysis step includes: a first calculation step of calculating an area of the entire spheroid in the photographic image; a second calculation step of calculating an area of the localization region in the photographic image; a third calculation step of calculating a ratio of the localization region on the basis of the area of the entire spheroid and the area of the localization region; and a fourth calculation step of calculating an evaluation parameter on the basis of the ratio of the localization region.

Effects of the Invention

[0013] According to the first to sixth inventions of the present application, the localization region in which a specific kind of cells are localized is extracted from the photographic image of the spheroid including multiple kinds of cells, and the evaluation parameter based on the ratio of the localization region can be calculated. This enables noninvasive evaluation of the spheroid.

[0014] Especially, according to the third invention and the fourth invention of the present application, a parameter that directly shows whether or not the spheroid exhibits a required behavior as a part of tissue is used as the evaluation parameter, which makes it possible to obtain an evaluation parameter that is easier for a user to grasp.

[0015] Especially, according to the fifth invention of the present application, a localization region corresponding to an unusual portion extending over the outer surface of the biological sample can be accurately extracted.

[0016] Especially, according to the sixth invention of the present application, a localization region can be accurately extracted from the photographic image of the spheroid by using the trained model.

[0017] These and other objects, features, aspects and advantages of the present invention will become more apparent from the following detailed description of the present invention when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. **1** is a diagram showing a configuration of an observation apparatus;

[0019] FIG. **2** is a control block diagram of the observation apparatus;

[0020] FIG. **3** is a block diagram conceptually showing functions of an image processing/evaluation apparatus;

[0021] FIG. **4**A is a view schematically showing an example of a spheroid;

[0022] FIG. **4**B is a view schematically showing another example of a spheroid;

[0023] FIG. **5** is a flowchart showing a flow from a formation step to a tissue formation step;

[0024] FIG. **6** is a view schematically showing a tomographic image;

[0025] FIG. **7** is a flowchart showing an example of a flow of a region extraction step of extracting a localization region;

[0026] FIG. **8** is a view schematically showing a result of extraction of an entire region;

[0027] FIG. **9** is a view schematically showing a result of extraction of a localization region;

[0028] FIG. **10** is a flowchart showing an example of a flow of an analysis and evaluation step for a spheroid;

[0029] FIG. **11** is a graph showing a result of Experiment 1;

[0030] FIG. **12** is a graph showing a result of Experiment 2; and

[0031] FIG. **13** is a flowchart showing a flow from a formation step to a tissue formation step in a case in which a trained model is used.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0032] Hereinafter, a preferred embodiment of the present invention will be described with reference to the drawings.

<1. Configuration of Observation Apparatus>

[0033] FIG. **1** is a diagram showing a configuration of an observation apparatus **1** according to one preferred embodiment of the present invention. The observation apparatus **1** is an apparatus that images a biological sample **9** held in a sample holder **90** and evaluates the condition of the biological sample **9** on the basis of the acquired image.

[0034] The biological sample **9** to be observed and evaluated by the observation apparatus **1** is a cell aggregate called a spheroid.

[0035] A spheroid can be put to known various uses. For example, by placing a plurality of spheroids in a bio 3D printer, it is possible to form a three-dimensional structure of a larger size. In placing spheroids in a bio 3D printer, it is preferable to three-dimensionally place the spheroids. For example, in a case in which a three-dimensional structure is formed by using spheroids in which two or more kinds of cells composing a liver are uniformly included, the formed three-dimensional structure can be put to uses in the field of regenerative medicine, evaluation of hepatotoxicity of a substance under test, and the like. Preferably, the observation apparatus **1** evaluates whether the condition of a spheroid including multiple kinds of cells is good or bad, in order to select a spheroid suitable as a material in a bio 3D printer.

[0036] As shown in FIG. **1**, the observation apparatus **1** includes a stage **10**, an imaging unit **20**, and a computer **30**.

[0037] The stage **10** is a support plate that supports the sample holder **90**. For the sample holder **90**, for example, a well plate is used. The well plate consists of multiple wells **91**. The well **91** is a recessed portion that is recessed downward. Each of the wells **91** has a U-shaped or V-shaped bottom. The biological sample **9**, together with a culture medium, is held near the bottom of each well **91**. For a material of the sample holder **90**, transparent resin or glass that transmits light is used.

[0038] The stage **10** includes an opening **11** that vertically penetrates. The sample holder **90** is horizontally supported while being fit in the opening **11** of the stage **10**. Thus, the lower surface of the sample holder **90** is exposed toward the imaging unit **20** without being covered by the stage **10**.

[0039] The imaging unit **20** is a unit that images the biological sample **9** in the sample holder **90**. The imaging unit **20** is placed below the sample holder **90** supported by the stage **10**. The imaging unit **20** of the present preferred embodiment is an optical coherence tomography (OCT) device capable of imaging the biological sample **9** to produce a tomographic image and a three-dimensional image thereof.

[0040] As shown in FIG. **1**, the imaging unit **20** includes a light source **21**, an object optical system **22**, a reference optical system **23**, a detection unit **24**, and an optical fiber coupler **25**. The optical fiber coupler **25** includes first to fourth optical fibers **251** to **254** connected at a connecting unit **255**. The light source **21**, the object optical system **22**, the reference optical system **23**, and the detection unit **24** are connected to each other via optical paths formed by the optical fiber coupler **25**.

[0041] The light source **21** includes a light emitting element such as an LED. The light source **21** emits low-coherence light including a wide range of wavelength components. In order to allow light to reach the inside of the biological sample **9** without invasively treating the biological sample **9**, it is desirable that light emitted from the light source **21** is a near-infrared ray. The light source **21** is connected to the first optical fiber **251**. Light emitted from the light source **21** is incident on the first optical fiber **251** and is separated into light incident on the second optical fiber **252** and light incident on the third optical fiber **253** at the connecting unit **255**.

[0042] The second optical fiber **252** is connected to the object optical system **22**. Light traveling from the connecting unit **255** to the second optical fiber **252** is incident on the object optical system **22**. The object optical system **22** includes a plurality of optical components including a collimator lens **221** and an object lens **222**. Light emitted from the second optical fiber **252** passes through the collimator lens **221** and the object lens **222**, and is applied to the biological sample **9** in the sample holder **90**. At that time, the object lens **222** causes the light to converge to the biological sample **9**. Then, light reflected from the biological sample **9** passes through the object lens **222** and the collimator lens **221** and is again incident on the second optical fiber **252**. Hereinafter, the light reflected from the biological sample **9** will be referred to as "observation light".

[0043] As shown in FIG. **1**, the object optical system **22** is connected to a scan mechanism **223**. The scan mechanism **223** slightly moves the object optical system **22** vertically and horizontally in accordance with an instruction from the computer **30**. Thus, the incidence position of light on the biological sample **9** can be slightly moved vertically and horizontally.

[0044] Further, the imaging unit **20** can be moved horizontally by a movement mechanism not shown. Thus, the field of view of the imaging unit **20** can be changed among the plurality of wells **91**.

[0045] The third optical fiber **253** is connected to the reference optical system **23**. Light travelling from the connecting unit **255** to the third optical fiber **253** is incident on the reference optical system **23**. The reference optical system **23** includes a collimator lens **231** and a mirror **232**. Light emitted from the third optical fiber **253** passes through the collimator lens **231** and is incident on the mirror **232**. Then, light reflected from the mirror **232** passes through the collimator lens **231** and is again incident on the third optical fiber **253**. Hereinafter, the light reflected from the mirror **232** will be referred to as "reference light".

[0046] As shown in FIG. **1**, the mirror **232** is connected to a retraction mechanism **233**. The retraction mechanism **233** slightly moves the mirror **232** in an optical-axis direction in accordance with an instruction from the computer **30**. Thus, the optical-path length of the reference light can be changed.

[0047] The fourth optical fiber **254** is connected to the detection unit **24**. The observation light that is incident on the second optical fiber **252** from the object optical system **22** and the reference light that is incident on the third optical fiber **253** from the reference optical system **23** join together at the connecting unit **255**, and are incident on the fourth optical fiber **254**. Then, light emitted from the fourth optical fiber **254** is incident on the detection unit **24**. At that time, interference is caused between the observation light and the reference light due to a phase difference therebetween. The optical spectrum of interference light at that time varies with the height of reflection position of the observation light.

[0048] The detection unit **24** includes a spectroscope **241** and a light detector **242**. The interference light emitted from the fourth optical fiber **254** is dispersed into each wavelength component in the spectroscope **241**, and is incident on the light detector **242**. The light detector **242** detects each dispersed interference light, and outputs its corresponding detection signal to the computer **30**.

[0049] An image acquisition unit **41** described later in the computer **30** performs Fourier transform on the detection signal provided from the light detector **242**, to thereby calculate a vertical light-intensity distribution of the observation light. Further, while the object optical system **22** is horizontally moved by the scan mechanism **223**, the image acquisition unit **41** repeats the above-described calculation of light-intensity distribution to thereby calculate a light-intensity distribution of the observation light at each coordinate position in a three-dimensional space. Consequently, the computer **30** can acquire a tomographic image and a three-dimensional image of the biological sample **9**.

[0050] A tomographic image is formed of a plurality of pixels arranged on two-dimensional coordinates, and is data in which each pixel has a predetermined intensity value. A three-dimensional image is formed of a plurality of voxels arranged on three-dimensional coordinates and is data in which each voxel has a predetermined intensity value. That is, each of a tomographic image and a three-dimensional image is a photographic image in which intensity values are distributed on predetermined coordinates.

[0051] The computer **30** functions as a control unit that controls the operation of the imaging unit **20**. Further, the computer **30** functions as a data processing unit that produces a tomographic image and a three-dimensional image on the basis of a detection signal received from the imaging unit **20** and evaluates the condition of the biological sample **9** on the basis of the acquired tomographic image and three-dimensional image.

[0052] FIG. **2** is a control block diagram of the observation apparatus **1**. As conceptually shown in FIG. **2**, the computer **30** includes a processor **31** such as a CPU, a memory **32** such as a RAM, and a storage unit **33** such as a hard disk drive. In the storage unit **33**, a control program P1 for controlling the operations of respective components in the observation apparatus **1** and a data processing program P2 for producing a tomographic image and a three-dimensional image and evaluating the condition of the biological sample **9**, are stored.

[0053] Further, as shown in FIG. **3**, the computer **30** is connected to the light source **21**, the scan mechanism **223**, the retraction mechanism **233**, and the light detector **242** that have been described above, and to a display unit **70** described later such that the computer **30** can conduct communication with each of those components. The computer **30** controls the operations of the above-described respective components in accordance with the control program P1. Thus, an imaging process of the biological sample **9** held in the sample holder **90** proceeds.

[0054] FIG. **3** is a block diagram conceptually showing the functions of the computer **30** serving as an image processing/evaluation apparatus for observing and evaluating the biological sample **9**. As shown in FIG. **3**, the computer **30** includes the image acquisition unit **41**, a region extraction unit **42**, and an evaluation unit **43**. The respective functions of the image acquisition unit **41**, the region extraction unit **42**, and the evaluation unit **43** are performed by an operation of the processor **31** of the computer **30** in accordance with the data processing program P2 described above. Details of processes performed by the image acquisition unit **41**, the region extraction unit **42**, and the evaluation unit **43** will be given later.

<2. Formation Step, Image Acquisition Step, Region Extraction Step, Analysis and Evaluation Step, and Tissue Formation Step>

[0055] Next, a formation step of the spheroid **9** (biological sample **9**) in the above-described observation apparatus **1**,

an image acquisition step, a region extraction step, an analysis and evaluation step, and a tissue formation step are described.

[0056] For the spheroid **9**, a spheroid including various kinds of cells including a main tissue, a nervous tissue, and the like can be used. Preferably, the spheroid **9** is a material used in a bio 3D printer.

[0057] In any spheroid including multiple kinds of cells, irrespective of which tissue is an origin of the cells included in the spheroid, the cells are not uniformly distributed, so that a specific kind of cells are localized in a part in some cases, which may probably affect gene expression or protein secretion of the spheroid. Hence, it is required to evaluate whether or not multiple kinds of cells are uniformly distributed in the spheroid **9**, preferably, whether or not multiple kinds of cells are distributed uniformly enough to be applied to formation of a structure for regenerative medicine using a bio 3D printer, or for in vitro evaluation, or the like.

[0058] The spheroid **9** can be obtained through co-culture of two or more kinds of cells by a known method in accordance with a requirement such as a purpose of use, a kind of cell, or a ratio.

[0059] The spheroid **9** is preferably a so-called liver spheroid. A liver spheroid is a cell aggregate including two or more kinds of cells including a liver parenchymal cell (hepatocyte) among multiple kinds of cells composing a liver. A liver spheroid can include any cell derived from a liver, in addition to a liver parenchymal cell (hepatocyte), and may include, for example, a hepatic sinusoidal endothelial cell, a Kupffer cell, a hepatic stellate cell, a pit cell, a bile duct epithelial cell, a mesothelial cell, and the like

[0060] FIGS. **4**A and **4**B are views schematically showing examples of the spheroid **9** that is an object to be observed in the following description. The spheroid **9** shown in each of FIGS. **4**A and **4**B includes a localization region **9***a*. The localization region **9***a* is a portion in which a specific kind of cells among multiple kinds of cells composing the spheroid **9** are localized.

[0061] For example, the localization region **9***a* is expected to be localized in the surface of (on the outside of) the spheroid **9** as shown in FIG. **4**A, or is expected to be localized in the inside of the spheroid **9** as shown in FIG. **4**B.

[0062] FIG. **5** is a flowchart showing a flow from the formation step to the tissue formation step. In performing the analysis and evaluation step for the spheroid **9** with the use of the observation apparatus **1** and performing the tissue formation step with the use of a bio 3D printer, first, many spheroids **9** are formed (step S**1**, spheroid formation step). Then, the formed spheroids **9** are put in the sample holder **90**. Note that the spheroids **9** may be formed within the sample holder **90**, or the spheroids **9** having been formed in a separate culture vessel may be put in the sample holder **90**. At that time, in the sample holder **90**, a plurality of spheroids **9**, together with a culture medium, are held.

[0063] Subsequently, the sample holder **90** is set on the stage **10** of the observation apparatus **1**, and the spheroids **9** are imaged with the use of imaging unit **20** (step S**2**, image acquisition step). In the present preferred embodiment, the imaging unit **20** images by optical coherence tomography. Specifically, the light source **21** is caused to emit light. Then, while the object optical system **22** is slightly moved by the scan mechanism **223**, each wavelength component of interference light of observation light and reference light is detected by the light detector **242**. The image acquisition unit **41** of the computer **30** calculates a light-intensity distribution in each coordinate position of the spheroid **9** on the basis of each detection signal output from the light detector **242**. Consequently, a tomographic image D1 and a three-dimensional image D2 of the spheroid **9** are acquired.

[0064] The observation apparatus **1** acquires a plurality of tomographic images D1 and one three-dimensional image D2 for one spheroid **9**. Further, the observation apparatus **1** repeats the process of the step S**2** while changing the well **91** to be imaged, to thereby acquire the tomographic images D1 and the three-dimensional images D2 of the plurality of spheroids **9**. The acquired tomographic images D1 and three-dimensional images D2 are stored in the storage unit **33** of the computer **30**. Moreover, the computer **30** displays the acquired tomographic images D1 and three-dimensional images D2 on the display unit **70**.

[0065] FIG. **6** is a view schematically showing the tomographic image D1. In the example of FIG. **6**, there is a region corresponding to the localization region **9***a* in the outer edge of the spheroid **9** having a substantially spherical shape. Hereinafter, the region corresponding to the localization region **9***a* will be referred to as a "localization region A". The region extraction unit **42** of the computer **30** extracts the localization region A from the tomographic image D1 (step S**3**, region extraction step).

[0066] FIG. **7** is a flowchart showing an example of a flow of the region extraction step of extracting the localization region **9***a* (localization region A) of the spheroid **9** in the step S**3**. In the example of FIG. **7**, first, the region extraction unit **42** extracts an entire region A1 corresponding to the entire spheroid **9** from the tomographic image D1 (step S**31**, first extraction step). Specifically, the region extraction unit **42** extracts a region having a intensity value higher than a first threshold value set in advance in the tomographic image D1, as the entire region A1. FIG. **8** is a view schematically showing a result of extraction of the entire region A1.

[0067] Secondly, the region extraction unit **42** extracts a region having an intensity value higher than a predetermined second threshold value from the entire region A1, as a high-intensity region A2 (step S**32**, second extraction step). Note that the second threshold value is higher than the first threshold value. In FIG. **8**, the high-intensity region A2 having a ring shape is indicated by a broken line. The outer edge of a cell aggregate having a substantially spherical shape has a strong tendency to have a high intensity. For this reason, in the step S**32**, the high-intensity region A2 is extracted, so that the outer edge of the cell aggregate having a substantially spherical shape is defined.

[0068] Subsequently, the region extraction unit **42** extracts a region that is left after the high-intensity region A2 and a region inner than the high-intensity region A2 are excluded from the entire region A1, as the localization region A (step S**33**, third extraction step). FIG. **9** is a view schematically showing a result of extraction of the localization region A. Thus, the localization region A corresponding to the localization region **9***a* extending over the outer surface of the cell aggregate having a spherical shape can be appropriately extracted.

[0069] In this manner, in the step S**3**, a region of which intensity value satisfies a predetermined requirement in the tomographic image D1 is extracted as the localization region A. The procedure for extracting the localization region A is not necessarily required to be the same as the above-described steps S**31** to S**33**. The region extraction unit **42** is

only required to extract the localization region A by following an appropriate procedure depending on the position or the intensity value of the localization region A.

[0070] After that, the evaluation unit **43** of the computer **30** analyzes the localization region A extracted in the step S**3**, to evaluate the condition of the spheroid **9** (step S**4**, analysis and evaluation step). In the step S**4**, the evaluation unit **43** analyzes the localization region **9***a* in each of the tomographic image D1 and the three-dimensional image D2, to evaluate the condition of the spheroid **9**.

[0071] FIG. **10** is a flowchart showing an example of a flow of the analysis and evaluation step of evaluating the condition of the spheroid **9** in the step S**4**. In the analysis and evaluation step in the example of FIG. **10**, the condition of the spheroid **9** is evaluated on the basis of the area of the localization region A in the tomographic image D1 that is a photographic image. Further, the analysis and evaluation step in the example of FIG. **10** includes an analysis step including steps S**41** to S**44** and an evaluation step including a step S**45**.

[0072] In the example of FIG. **10**, first, the evaluation unit **43** calculates the area of the entire spheroid **9** in the tomographic image D1 (step S**41**, first calculation step). Specifically, the evaluation unit **43** calculates the area of the entire region A1 in the tomographic image D1. The area of the entire region A1 can be calculated on the basis of the number of pixels included in the entire region A1, for example.

[0073] Secondly, the evaluation unit **43** calculates the area of the localization region A in the tomographic image D1 (step S**42**, second calculation step). Specifically, the evaluation unit **43** calculates the area of the localization region A in the tomographic image D1. The area of the localization region A can be calculated on the basis of, for example, the number of pixels included in the localization region A.

[0074] Subsequently, the evaluation unit **43** calculates the ratio of the localization region **9***a* on the basis of the area of the entire spheroid **9** calculated in the first calculation step of the step S**41** and the area of the localization region A calculated in the second calculation step of the step S**42** (step S**43**, third calculation step). The ratio calculated in the step S**43** may be, for example, an area ratio in one representative tomographic image D1, that is, a result of division of the area of the localization region A by the area of the entire region A1.

[0075] Meanwhile, for example, in a case in which a plurality of tomographic images D1 are acquired for one spheroid **9**, the ratio of the localization region **9***a* calculated in the third calculation step of the step S**43** may be a volume ratio. In this case, the respective volumes of the entire spheroid **9** and the localization region **9***a* are calculated on the basis of the respective areas of the entire region A1 and the localization region A in each of the plurality of tomographic images D1, first. Then, a ratio of the localization region **9***a* is calculated by division of the volume of the localization region **9***a* by the volume of the entire spheroid **9**.

[0076] After that, the evaluation unit **43** calculates the evaluation parameter V (step S**44**, fourth calculation step). The evaluation parameter V is calculated on the basis of the ratio of the localization region **9***a* calculated in the third calculation step of the step S**43**. The evaluation parameter V is, for example, an estimated value of a degree of predetermined gene expression or an estimated value of a secretion amount of predetermined protein in the spheroid **9** as shown in Experiment 2 described later. The estimated value in either case is calculated on the basis of the ratio of the localization region **9***a*. In a case in which the spheroid **9** is a liver spheroid, an amount of protein CYP3A4 secreted from the spheroid **9** is used as the evaluation parameter V, for example.

[0077] As the evaluation parameter V, the ratio of the localization region **9***a* calculated in the third calculation step of the step S**43** may be directly used. However, by using, as the evaluation parameter V, a parameter that directly shows whether or not the spheroid exhibits a required behavior as a part of tissue, such as an estimated value of a degree of predetermined gene expression or an estimated value of a secretion amount of predetermined protein, it is possible to obtain the evaluation parameter V that is easier for a user to grasp.

[0078] The evaluation unit **43** determines the quality of the spheroid **9** on the basis of the calculated evaluation parameter V (step S**45**, determination step). For example, when the evaluation parameter V is equal to or larger than a predetermined threshold value, or is equal to or smaller than a predetermined threshold value, it is determined that the spheroid **9** is suitable as a material in a bio 3D printer. Otherwise, it is determined that the spheroid **9** is not suitable as a material in a bio 3D printer.

[0079] For example, in a case in which the evaluation parameter V is exactly equal to a ratio of a localization region and the purpose is to select a healthy spheroid suitable for regenerative medicine or hepatotoxicity evaluation, it is preferable to determine a spheroid of which evaluation parameter V is equal to or smaller than the predetermined threshold value as "excellent", and determine a spheroid of which evaluation parameter V is larger than the predetermined threshold value as "unacceptable".

[0080] In a case in which an estimated value of a degree of predetermined gene expression or an estimated value of a secretion amount of predetermined protein in a spheroid, calculated on the basis of a ratio of the localization region **9***a*, is used as the evaluation parameter V, it is preferable to determine a spheroid of which evaluation parameter V is equal to or larger than a predetermined lower-limit threshold value and is equal to or smaller than a predetermined upper-limit threshold value, as "excellent", depending on the kind of a gene or protein, the composition of the spheroid, the purpose of use of the spheroid, or the like.

[0081] The size (area or volume) of the localization region **9***a*, the ratio of the localization region **9***a*, and the evaluation parameter V that are calculated by the evaluation unit **43** are stored in the storage unit **33**. Further, the evaluation unit **43** displays the evaluation parameter V on the display unit **70**.

[0082] As described above, in the observation apparatus **1**, the spheroid **9** is imaged by optical coherence tomography, and the localization region A corresponding to the localization region **9***a* in the spheroid **9** can be extracted from the acquired tomographic image D1. This enables noninvasive observation of the localization region **9***a* of the spheroid **9**. Further, the evaluation parameter V based on the ratio of the localization region **9***a* is calculated, which makes it possible to easily evaluate whether the spheroid **9** is suitable or non-suitable as a material in a bio 3D printer.

[0083] The image acquisition step of the step S**2**, the region extraction step of the step S**3**, and the analysis and evaluation step of the step S**4** described above are performed

on many spheroids **9** having been formed. Then, the spheroid **9** that has been determined as a suitable material in a bio 3D printer in the analysis and evaluation step of the step S**4** is used to form tissues with the use of, for example, a bio 3D printer (step S**5**, tissue formation step). In this manner, high-quality tissues suitable for use in, for example, evaluation of hepatotoxicity, can be obtained.

3. EXPERIMENTS

3-1. Experiment 1

[0084] In order to confirm the accuracy of detecting a localization region in a tomographic image (hereinafter referred to as an "OCT image") acquired by optical coherence tomography in the evaluation method of the above-described preferred embodiment, an area ratio of a localization region in a liver spheroid was calculated by the following two methods.

(First Method: Calculating Area Ratio of Localization Region from OCT Image)

[0085] A plurality of liver spheroids were imaged using optical coherence tomography by the method described in the above-described embodiment, and OCT images (tomographic images) were acquired. A localization region was extracted from a randomly-selected image out of the acquired OCT images, and a value obtained by division of the localization region by an entire region was calculated as an area ratio.

(Second Method: Calculating Area Ratio of Localization Region from Stained Tissue-Section Image)

[0086] The plurality of liver spheroids having been imaged using optical coherence tomography by the first method were stained using a fluorescent reagent, and were each cut along a randomly-selected section. In the cut surface, a localization region was extracted by the conventional method, and a value obtained by division of the localization region by an entire region was calculated as an area ratio.

[0087] FIG. **11** shows a result of Experiment 1, and is a graph showing a relationship between the area ratio of the localization region calculated from the image (OCT image) acquired by optical coherence tomography and the area ratio of the localization region calculated from the stained tissue-section image, for the liver spheroids.

[0088] As shown in FIG. **11**, Pearson's product-moment correlation coefficient between the area ratio of the localization region calculated from the OCT image acquired by optical coherence tomography and the area ratio calculated from the stained tissue-section image according to the conventional method was 0.839. That is, the area ratio of the localization region calculated from the OCT image acquired by optical coherence tomography has an adequate correlation with the area ratio calculated from the stained tissue-section image according to the conventional method. Therefore, it can be considered that the method of calculating an area ratio of a localization region from an OCT image acquired by optical coherence tomography can calculate an area ratio with accuracy similar to that of the conventional method.

3-2. Experiment 2

[0089] In order to confirm the reliability of an estimated value for the evaluation parameter V in a case in which an estimated value of a degree of predetermined gene expression or an estimated value of a secretion amount of predetermined protein is used as the evaluation parameter V in the evaluation method of the above-described preferred embodiment, an experiment was conducted by the following method.

[0090] First, a plurality of liver spheroids were imaged using optical coherence tomography by the method described in the above-described preferred embodiment, and OCT images (tomographic images) were acquired. A localization region was extracted from a randomly-selected image out of the acquired OCT images, and a value obtained by division of the localization region by an entire region was calculated as an area ratio.

[0091] Meanwhile, the enzyme activity of protein CYP3A4 secreted from each of the plurality of liver spheroids having been imaged by optical coherence tomography was measured. Note that, for each of the liver spheroids, either imaging by optical coherence tomography or measurement of the enzyme activity of protein CYP3A4 may be performed earlier.

[0092] FIG. **12** shows a result of Experiment 2, and is a graph showing a relationship between the area ratio of the localization region calculated from the OCT image and the enzyme activity of CYP3A4, for the liver spheroids. As shown in FIG. **12**, Pearson's product-moment correlation coefficient between the area ratio of the localization region in each liver spheroid, calculated from the OCT image, and the enzyme activity of CYP3A4, was −0.885. Between the area ratio of the localization region in each liver spheroid, calculated from the OCT image, and the enzyme activity of CYP3A4, a high (negative) correlation can be found. Therefore, it can be said that, when the enzyme activity of CYP3A4 is estimated on the basis of an area ratio of a localization region calculated from an OCT image for a liver spheroid, the resultant estimated value has sufficient reliability.

<4. Modifications>

[0093] Hereinabove, one preferred embodiment of the present invention has been described, but the present invention is not limited to the above-described preferred embodiment.

<4-1. First Modification>

[0094] According to the above-described preferred embodiment, in the step S**3**, the region extraction unit **42** extracts a region of which intensity value satisfies a predetermined requirement in the tomographic image D1, as the localization region A. Alternatively, in the step S**3**, the region extraction unit **42** may extract the localization region A by using a trained model. FIG. **13** is a flowchart showing a flow of an evaluation process in a case in which a trained model is used.

[0095] In the example of FIG. **13**, first, the computer **30** creates a trained model by deep learning (step S**0**, training step). The trained model is an input/output device configured to receive the tomographic image D1 of the spheroid **9** as input information and produce the localization region A corresponding to the localization region **9***a* in the tomographic image D1 as output information. The computer **30** creates the trained model in accordance with a supervised machine learning algorithm by using a number of the

tomographic images D1 prepared in advance and the localization regions A included in the tomographic images D1, as training data. The trained model is stored in the storage unit **33**. For the machine learning algorithm, semantic segmentation can be used, for example, but the machine learning algorithm is not limited thereto.

[0096] Meanwhile, many spheroids **9** are formed (step S**1**, spheroid formation step). The spheroid formation step of the step S**1** may be performed after the training step of the step S**0** as shown in FIG. **13**, or may be performed before the training step or in parallel with the training step.

[0097] After the training step of the step S**0** and the spheroid formation step of the step S**1** are performed, the observation apparatus **1** take an image of the spheroid **9** (step S**2**, image acquisition step). Subsequently, the region extraction unit **42** of the computer **30** extracts the localization region A corresponding to the localization region **9***a* from the tomographic image D1 acquired by imaging (step S**3**, region extraction step). Specifically, the region extraction unit **42** inputs the tomographic image D1 acquired in the step S**2** to the trained model created in the step S**0**. As a result of this, the localization region A is output from the trained model. Thus, it is possible to extract the localization region A without performing processes such as the steps S**31** to S**33** described above.

<4-2. Second Modification>

[0098] The above-described preferred embodiment has discussed a case in which the localization region A is extracted from the tomographic image D1. Alternatively, the localization region A may be extracted from the three-dimensional image D2. In a case in which the three-dimensional image D2 is used for region extraction, a three-dimensional region corresponding to the localization region **9***a* is extracted from three-dimensional coordinates forming the three-dimensional image D2, as the localization region A, in the step S**3** described above. Then, the ratio of the localization region **9***a* is calculated from the volume of the extracted three-dimensional region corresponding to the localization region **9***a* and the volume of the entire spheroid **9**, and the evaluation parameter V is calculated on the basis of the ratio. The extraction method in this case may be region extraction based on an intensity value, like that in the above-described preferred embodiment, or may be region extraction using a trained model, like that in the above-described first modification.

<4-3. Other Modifications>

[0099] According to the above-described preferred embodiment, the sample holder **90** is a well plate including the plurality of wells (recessed portions) **91**, and each of the wells **91** holds one biological sample **9**. Alternatively, one well may hold a plurality of biological samples. In such a case, one image may include regions respectively corresponding to the plurality of biological samples. Further alternatively, the sample holder that holds a biological sample may be a dish having only one recessed portion.

[0100] Moreover, the respective elements described in the above-described preferred embodiment and modifications may be appropriately combined unless contradiction occurs.

[0101] While the invention has been shown and described in detail, the foregoing description is in all aspects illustrative and not restrictive. It is therefore understood that numerous modifications and variations can be devised without departing from the scope of the invention.

What is claimed is:

1. An evaluation method for a spheroid used in a bio 3D printer, comprising:

an image acquisition step of imaging the spheroid by optical coherence tomography and acquiring a photographic image;

a region extraction step of extracting a localization region in which one kind of cell is localized, from the photographic image;

an analysis step of analyzing the localization region; and

an evaluation step of evaluating a condition of the spheroid, wherein

the spheroid is a spheroid including multiple kinds of liver-derived cells including a hepatocyte, and

the analysis step includes:

a first calculation step of calculating an area of the entire spheroid in the photographic image;

a second calculation step of calculating an area of the localization region in the photographic image;

a third calculation step of calculating a ratio of the localization region on the basis of the area of the entire spheroid and the area of the localization region; and

a fourth calculation step of calculating an evaluation parameter on the basis of the ratio of the localization region.

2. The evaluation method according to claim **1**, wherein the evaluation step includes a determination step of determining a quality of the spheroid on the basis of the evaluation parameter.

3. The evaluation method according to claim **1**, wherein the evaluation parameter is an estimated value of a secretion amount of predetermined protein in the spheroid.

4. The evaluation method according to claim **3**, wherein the evaluation parameter is an estimated value of enzyme activity of CYP3A4 in the spheroid.

5. The evaluation method according to claim **1**, wherein the region extraction step includes:

a first extraction step of extracting an entire region corresponding to the spheroid, from the photographic image;

a second extraction step of extracting a high-intensity region of which intensity value is higher than a predetermined threshold value in the entire region; and

a third extraction step of extracting a region that is left after the high-intensity region and a region inner than the high-intensity region are excluded from the entire region, as the localization region.

6. An evaluation method for a spheroid used in a bio 3D printer, comprising:

a training step of creating a trained model configured to receive a photographic image of the spheroid imaged by optical coherence tomography, as input information, and produce a localization region in which one kind of cell is localized, as output information, by deep learning;

an image acquisition step of imaging the spheroid by optical coherence tomography and acquiring a photographic image;

a region extraction step of inputting the photographic image to the trained model, to extract the localization region output from the trained model;

an analysis step of analyzing the localization region; and

an evaluation step of evaluating a condition of the spheroid, wherein

the spheroid is a spheroid including multiple kinds of liver-derived cells including a hepatocyte, and

the analysis step includes:

a first calculation step of calculating an area of the entire spheroid in the photographic image;

a second calculation step of calculating an area of the localization region in the photographic image;

a third calculation step of calculating a ratio of the localization region on the basis of the area of the entire spheroid and the area of the localization region; and

a fourth calculation step of calculating an evaluation parameter on the basis of the ratio of the localization region.

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