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Biotechnology — Cellular morphological analysis — General requirements and considerations for cell morphometry to quantify cell morphological features

Biotechnologie — Analyse morphologique cellulaire — Exigences générales et considérations pour la morphométrie cellulaire afin de quantifier les caractéristiques morphologiques des cellules

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Foreword

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This document was prepared by Technical Committee ISO/TC 276, *Biotechnology*, Subcommittee SC 1, *Analytical methods.*

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Optical microscopy is a widely practiced technique for characterization of processed cells. Morphology of cells, such as shape, size, texture of whole or parts of cells, can provide information about various aspects of cells including identity, phenotype, viability, doubling time, as well as the states of stress and drug responses. Morphological evaluation of cells is widely employed in basic research, drug discovery, in-process control and release testing for cell manufacturing and cell banks for cell-based therapeutic products.

Therefore, it is desired to establish a common understanding of definitions and formulae regarding cell morphological descriptors in which specialists in research and business fields can refer to and compare information, within an institution, and with other interested parties.

The current situation is that characteristics of cell morphology obtained from microscopic images are frequently described qualitatively in expressions such as "unevenness around", "elongated", "rounded". Even when cell morphological descriptors characterizing the morphology of the cell are measured and quantified from the cell image, these cell morphological descriptors are not consistently used.

This document allows to check whether the numerical values assigned as cell morphological descriptors are calculated by an appropriate method, and to improve the reliability of the measured value and the evaluation result. It is expected that the "common language of definitions and mathematical formulae" based on this document will enable the accumulation of more reliable data, and such language will provide a basis for assessment whether individually acquired data can be quantitatively compared to each other.

This document is intended primarily for users, both in academia and industry, who evaluate cell characteristics. However, it can also be referred to by suppliers of tools such as microscopes, image processing devices, and software, suppliers of database that handle information on cell morphology and users who write scientific papers regarding cell morphology.

Biotechnology — Cellular morphological analysis — General requirements and considerations for cell morphometry to quantify cell morphological features

1 Scope

This document provides general requirements for cell morphometry to quantify cell morphological features including cell shape, size and texture.

This document addresses aspects of cell image capture using optical microscopy and image processing for morphometry.

This document does not address the statistics associated with a morphological analysis of a cellular sample.

This document also gives terms and definitions corresponding to cell morphological descriptors, and lists examples and their formulae, that represent quantitative cellular morphological features for evaluation of cell morphology in cell analysis.

This document primarily applies to morphological analysis of cultured mammalian cells.

This document is not intended for imaging used in clinical diagnostics.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
aberration
<optical system> failure of an optical system to produce a perfect image

EXAMPLE Spherical aberration, chromatic aberration

[SOURCE: ISO 10934:2020, 3.1.4, modified — "objective lens aberration" removed a preferred term and examples added.]

3.2
bit depth
maximum number of discrete levels available for the digitized representation of the signal intensity, represented as a power of 2

[SOURCE: ISO 22493:2014, 5.2.2.2.1, modified — "colour depth" and "pixel depth" are deleted from the preferred term, Notes 1 and 2 to entry are deleted.]

3.3
bounding box
rectangular region enclosing annotated object

Note 1 to entry: The major and minor axes of the rectangle are parallel to the edges of the images.

[SOURCE: ISO/IEC 30137-4:2021, modified — second sentence of Note 1 to entry deleted.]

3.4
cell morphology
form and structure of either the cell, subcellular components, or both

Note 1 to entry: In view of *cell morphometry* (3.6), cell morphology can be represented by a single or multiple *cell morphological descriptor(s)* (3.5) associated with *morphological feature(s)* (3.15).

3.5
cell morphological descriptor
quantitative representation of *cell morphology* (3.4)

3.6
cell morphometry
process of measuring dimensional, shape, and structural characteristics of cells including analysis of derived properties

Note 1 to entry: Steps for cell morphometry starts with determination of a purpose of the cell morphology analysis and ends with analysis of quantified results for the intended purpose. By taking these steps, cell morphometry can derive properties, e.g. phenotype such as immunosuppressive activity. See Table 1 for details.

3.7
cell shape
external geometric form of a cell

[SOURCE: ISO/TR 13014:2012 2.26, modified — term "shape" has been changed to "cell shape" and "particle" has been changed to "cell".]

3.8
cell texture
spatial arrangement of colours or intensities in an image associated with cellular characteristics

Note 1 to entry: The pattern can have a specific spatial scale or colour.

Note 2 to entry: The cell texture can result from a specific arrangement of sub-cellular components.

3.9
convex hull
smallest convex set containing a given geometric object

Note 1 to entry: "convex set" is a geometric set where any line segment connecting two points in the set lies entirely within the set.

[SOURCE: ISO 19107:2019, 3.15, modified – Note 1 to entry replaced.]

3.10
depth of focus
axial depth of the space on both sides of the image within which the image appears acceptably sharp, while the positions of the object plane and of the objective are maintained

[SOURCE: ISO 10934:2020, 3.1.37, modified — Note 1 to entry is deleted.]

3.11
formula
recipe for calculating a value

[SOURCE: ISO/IEC 29500-1:2016, 12.1.7, modified — the explanatory statement has been deleted.]

3.12
field of view
FOV
field which is observed by the microscope

Note 1 to entry: The full image frame of a digital imaging device corresponds to its field of view.

[SOURCE: ISO 13322-1:2014, 3.1.6, modified — the term “viewing” has been changed to “microscope”.]

3.13
image capture
image acquisition
process of creating a two-dimensional original image of an object

[SOURCE: ISO 21227-1:2003, 3.4]

3.14
measurement
process of experimentally obtaining one or more quantity values that can reasonably be attributed to a quantity

[SOURCE: ISO/IEC GUIDE 99:2007, 2.1, modified — Notes to entry have been deleted.]

3.15
morphological feature(s)
shape, size, and texture of cellular components

3.16
numerical aperture
NA
number originally defined by Abbe for objectives and condensers, which is given by the expression $n \sin u$, where n is the refractive index of the medium between the lens and the object and u is half the angular aperture of the lens

Note 1 to entry: Unless specified by “image-side”, the term refers to the object side.

[SOURCE: ISO 10934:2020, 3.1.10.4]

3.17
optical resolution
numerical measure of the image quality of an optical system

[SOURCE: ISO 8600-5:2020, 3.12]

3.18
pixel resolution
number of imaging pixels per unit distance of the detector

[SOURCE: ISO 15253:2021, 3.7, modified —hyphen between “pixel” and “resolution” was deleted.]

3.19
region of interest
ROI
parts of an image to which discrete observations are applied

Note 1 to entry: Region is selected by observer’s intended purpose.

[SOURCE: ISO 10934:2020, 3.2.28, modified — Note 1 to entry has been added.]

3.20
segmentation
partitioning images into distinct regions

Note 1 to entry: A distinct region is determined in attention of target of interest.

Note 2 to entry: The partitioning process includes filter application to the image.

Note 3 to entry: Segmentation can be of individual pixels. In such case, segmentation means partitioning pixels within images into distinct groups.

3.21
spatial resolution
smallest separation between two details in the object for which they can be detected as being separate under a given set of conditions

[SOURCE: ISO 15253:2021, 3.7, modified – "recognized" was replaced to "detected".]

3.22
tile capture
capturing method to extend a field of view by recording a series of tile images with limited field size

Note 1 to entry: Tile images can be recorded by systematically changing the relative position between the sample and the objective lens by mechanical drive.

Note 2 to entry: If tiles overlap, the tile images can be stitched to a larger overview image based on motor position or tile image correlation.

3.23
time lapse capture
image-recording method, in which multiple images are captured at specific time interval

Note 1 to entry: time lapse capture can be used for tracing changes in cell states or activities (such as cell division, fusion or phagocytosis of processed cells).

3.24
target of interest
TOI
part, region or both of cell(s) for morphological examinations defined by observer's intended purpose

3.25
Z-stack capture
image-recording method, in which multiple images are captured in the direction of the optical axis at a selected distance interval

Note 1 to entry: Z-stacks can be used to create a 3D image or for capturing objects in different optical focal planes.

4 Abbreviations

DIC differential interference contrast

FOV field of view

NA numerical aperture

TOI target of interest

ROI region of interest

5 General Concept

5.1 Cell morphometry

Cell morphology represents various features and states of cells, such as cell division and apoptosis, depending on the life cycle of the cell and environmental factors such as media contents and culture vessel. Cell morphometry can provide information on phenotype, viability, proliferation, stages of differentiation, function, and other cell characteristics. In addition, time-lapse observation of cell morphology can characterize dynamic properties of cells such as migration ability. It can also reflect a stimulatory response in a living biological system, including dynamic measurements of cellular morphology as an indication of toxicity in drug screening[6].

NOTE There are cases where acquiring 3D-images and time series images includes specific sample preparation and device handling, which are not covered in this document[7-9].

5.2 Steps for cell morphometry

Table 1 describes the steps for cell morphometry.

Table 1 — Steps for cell morphometry

Step#	Summary	Relevant clause
Step-1	Determine purpose of the cell morphology analysis	- (out of scope)
Step-2	Define appropriate TOI(s) for the intended purpose	6
Step-3	Select observation methods and sample preparation according to the TOI(s)	7.1
Step-4	Select or establish microscope system	7.2, 7.3
Step-5	Adjust settings of microscope system in order to acquire images	7.2, 7.3
Step-6	Perform segmentation (including pre/post-image processing) of the TOI(s) from acquired images	8
Step-7	Select appropriate cell morphological descriptors which characterize segmented TOI(s) and determine their numerical values	9, 10
Step-8	Preparation of a report for results of morphometric analysis	11
Step-9	Analysis with quantified results for the intended purpose	(out of scope)

6 Target of interest (TOI)

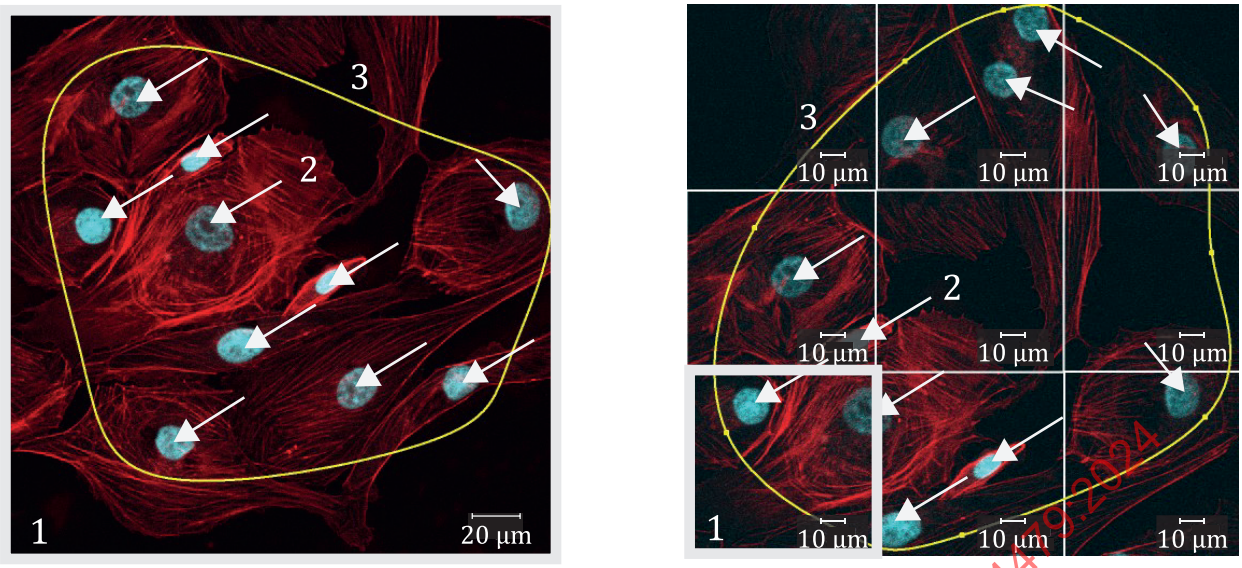
The TOI should be defined according to intended purpose of the morphology analysis.

The TOI should be defined before conducting the image capture step. This is important to select observation methods, microscope components and image capture devices, and their settings. Imaging conditions can be optimized by visual observation.

Images should not be optimized for human perception but for downstream image processing and analysis.

NOTE When the image capture conditions are determined by human perception, and when the image is checked after capture, the brightness, capture position, focus, image resolution, and other conditions can vary, which makes it difficult to analyse the image.

Figure 1 describes the relationship between FOV, TOI, and ROI.



a) Relationship between FOV, ROI, and TOI in a single image made of a single FOV

b) Relationship between FOV, ROI, and TOI in a tiled image made of 9 FOVs

Key

1 FOV
2 TOI
3 ROI

NOTE For the purpose of Figure 1, TOI is cell nucleus, and ROI is set to include TOIs.

Figure 1 — Relationship between FOV, TOI, and ROI

7 Image capture

7.1 Microscopic observation method

7.1.1 General

Optical microscopy is one of the most widely applied method for cell observation. Manual microscopy has been used in the past but is currently replaced by digital imaging and use of information technology[10].

In order to observe cells, users should consider the contrast of their sample and select methods (and microscope system) that support sufficient contrast and resolution for imaging.

A contrast-enhancing technique for optical microscopy should be selected so that the TOI can be visualized in sufficient contrast for the intended purpose prior to initiating measurements. Contrast-enhancing techniques that utilize difference in refractive indices, such as phase contrast or differential interference can be used. Staining (labelling, dyeing) can also be used. In addition, a method combining transmitted light observation and digital image processing can be used. Users should be aware that those techniques can influence morphometric measurements.

A microscope system including its components should be selected so that the TOI can be visualized in sufficient spatial and temporal resolution for the intended purpose.

NOTE 1 Understanding the dimension of the TOI helps the proper selection and settings for microscope system.

NOTE 2 Proper system and its adjustments of settings can be selected using predetermined reference materials for visual observation, such as positive/negative control cells or cell images. The points to consider for the selection are listed in 7.1.2. A summary is given in Table A.1.

NOTE 3 Points to consider when acquiring phase-contrast images of cells suitable for image analysis are described in Annex E. Checklists for adherent cells that do not form colonies, adhesive cells that form colonies, and spheroids are given in Tables E.1, E.2, and E.3, respectively.

The microscopic observation method shall be documented.

7.1.2 Cell properties to be observed

7.1.2.1 Adherence and suspension

Microscopic observation method should be selected while taking the cell culturing condition (e.g adherence, floating, or suspension) into account.

Cells adhered to the vessel wall tend to form a thin layer(s). Forming of a thin layer(s) decreases contrast of these cells. Therefore, appropriate contrast-enhancing techniques should be applied (see Table A.1). Assuming a flat homogenous substrate/image field, change in the location of cells during observation has little impact on the quality of the acquired image of these cells.

Since cells suspended in the culture medium can change their locations during the observation, the observation techniques should be selected in consideration of the temporal resolution.

7.1.2.2 Stack and stratification

Layering, stratification, and three-dimensional (3D) structuring of cells can affect cell observation.

NOTE 1 Most transmission-type methods are not adequate for observation of deep 3D structures because the illumination light is scattered, and less light is transmitted through the sample. Imaging with infrared light can reduce the influence of scattered light.

NOTE 2 When single-layer or multi-layer cells have significant variations in cell-layer thickness and height, a "halo" image effect appears around the cells. This effect lowers image quality in phase-contrast observation.

NOTE 3 For fluorescence-type measurements, light emitted from cell outside the imaging plane can reduce image quality. This problem can be reduced using confocal microscopy or related techniques. It is important to be aware that changing of microscope type can change performance characteristics/traceability.

7.1.2.3 Intracytoplasmic structures

Intracytoplasmic structures (such as pigments, granules, and vacuoles) can affect image capture. Intracytoplasmic structures increase cell contrast, therefore cells with sufficient amount of intracytoplasmic structures can be observed not only by phase-contrast, differential-interference methods but also by other transmitted light methods such as a brightfield.

7.1.3 Sample preparation

7.1.3.1 Sample preparation - general

Cell cultures are affected by the surrounding environment e.g., ambient temperature, humidity, CO_2 concentration. Changes in those conditions can lead to morphological changes of the cells up to cell death, in some cases. Therefore, care should be taken that the observation environment is equivalent to the culture environment, and that the cells do not undergo morphological changes during observation.

In addition, since morphological features of the cell changes depending on cell density and passages during culture, care should be taken to use experimental conditions which fit the intended purpose.

In the case when a portion of cells is taken out from the cell population and used for observation, it should be considered depending on the intended purpose, whether that portion includes the cells to be observed or whether that portion is representative of the whole cell population. The intended purpose should be described.

In the case when a portion of cells is transferred from the cell population to other vessels, procedures, materials such as type of vessels and pipetting devices, and reagents applied for the transfer should be properly selected.

Methods that can be applied for the observation depend on whether the observed or image-acquired cells are to be discarded, continued in culture for further observation, or used for other purposes.

Procedure and condition of sample preparation shall be documented.

7.1.3.2 Sample preparation for specific observation - Labelling, dyeing, and chemical treatments

7.1.3.2.1 General

Histochemical staining, fluorescent labelling, immunostaining, or other chemical treatments can contribute to TOI determination. Therefore, appropriate reagents and absorption/excitation wavelength and microscope components should be selected. Histochemical staining, fluorescent labelling, immunostaining, or other chemical treatments can alter cell membrane and nuclear properties, as well as other cell characteristics. As a result, the morphological characteristics of the treated cells can be different from those of untreated living cells.

If cells are to be used after microscopic observation, a processing method that minimally interferes with the intended use shall be applied.

7.1.3.2.2 Cell fixation

When fixing cells using a chemical fixative, a method that preserves cellular structures of interest should be applied.

NOTE Chemical treatments such as cell fixation can alter cell membrane and nucleus properties, as well as other cell characteristics. As a result, morphological features of treated cells can differ from those of living, untreated cells. Further guidance can be found in ISO 20166-4.

7.1.3.2.3 Fluorescent labelling or immunostaining

When applying a staining process, the intensity of staining should be sufficient to allow the signal to be detected over background.

NOTE 1 The term “detection” in the above sentence includes detection by visual observation and that by using an image capture device. There are cases where subvisible dyes, i.e. dyes that are not seen with visual observation, can be detected by using image capture device.

NOTE 2 If the light intensity is increased as a countermeasure to the insufficient staining, autofluorescence or phototoxicity can occur.

Non-specific staining can occur depending on the nature of the labelling or dyeing procedure, e.g., concentration of the reagents used.

Some types of staining also affect cell characteristics, making it impossible to continue culturing after observation and imaging.

Elapsed time and culture environment can affect cell activity and sensitization during observation or cause a photochemical change of the stained material.

Labels used for immunostaining or other fluorescent labelling shall be documented. This should include their excitation and emission characteristics.

If there is an intention to continue the cell culture after cell staining, the effect of cell staining on the culturability of the cells should be considered.

7.1.3.3 Observation vessels

The material and design of vessel applied for microscopy can affect the observation of cells.

In the case when same vessel is used both for cell culture and observation, its material and surface treatment should be suitable for both purposes.

On the other hand, there are cases that culturing is performed applying vessels which are incompatible for microscopy. When vessel is changed for observation in such cases, it is necessary to make sure that the state and morphological features of the cells are not affected by transportation between the vessels.

NOTE 1 The observation vessel is sometimes referred to as the sample holder, where the sample holder is the device (or sample carrier, container or vessel) on which the specimen was mounted, cultured, or adhered for microscopical examination. It is typically flat and can be made of glass or plastic (e.g., microscope slides, petri dishes, and multi-well plates)[11,12].

NOTE 2 Vessels made of polymeric material are often not appropriate for differential interference microscopy because of birefringence.

A vessel whose thickness entering the optical path is less than the working distance should be used.

NOTE 3 Working distance, i.e., the distance from a front lens element of the objective to a target of interest when the target of interest is in focus, depends on the optical design of the objective, such as magnification and NA.

7.1.3.4 Contamination

In the case when the cell sample is to be applied for subsequent culture after observation, measures should be taken to avoid contamination with microorganisms during the entire process from sample extraction, preparation, observation, and return to the culture environment.

In cases where cells cannot be automatically observed in the culture environment, it is recommended that a sample be removed from the culture, if possible, to avoid contamination of cells that will continue to undergo culturing.

7.2 Microscope system and its settings

7.2.1 General

Microscope system consists of light source, objective lens, image capture device, stage and a set of other filters and mirrors. The configuration of the instrument depends on the specific purpose of observation.

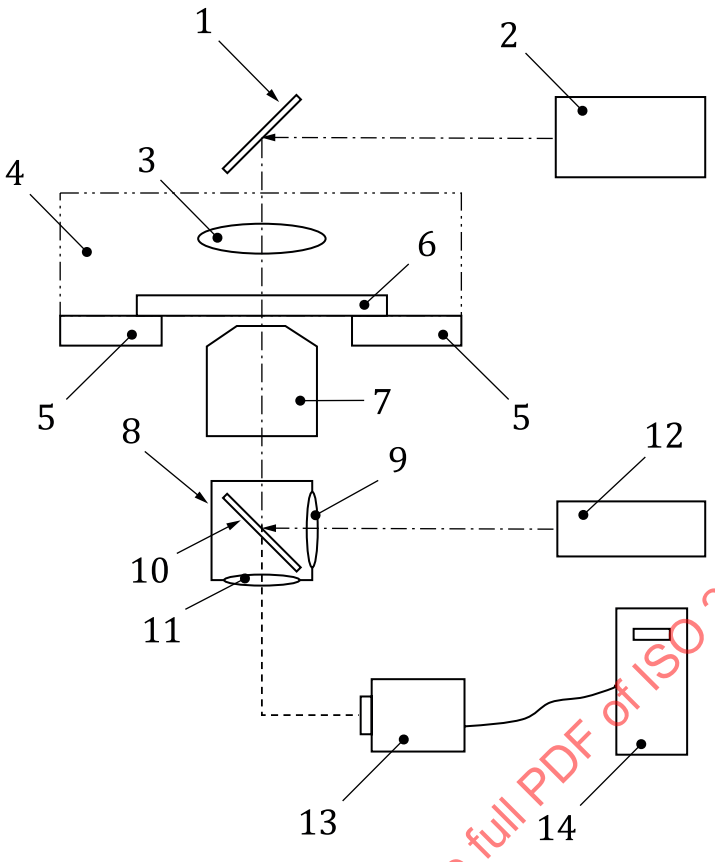
NOTE 1 This document presupposes that the microscope system is maintained properly.[13] For this, several resources exist. For example, traceable microscopic rulers and calibrated graticules can be used to calibrate size measurements[4,14]. Normalizations for non-uniformity of the field can be applied[3,5].

The spatial resolution of the image is determined by the combined optical resolution of the lens and the pixel resolution of the image capture device. Captured image of the ROI needs sufficient spatial resolution. The camera should have the pixel resolution necessary to achieve it.

NOTE 2 The optical resolution of the lenses depends on its NA. The camera resolution is determined by the size of the pixel and magnification.

Points to consider for each component depend on specific microscopic observation method.

The points to consider when selecting a microscope system and its settings are given in 7.2.2 to 7.2.9. A summary is given in Table B.1.



Key

1 mirror
2 light source
3 condenser lens
4 environment
5 stage
6 sample
7 objective lens
8 set of fluorescent filters
9 excitation filter
10 dichroic mirror
11 fluorescence filter
12 light source
13 image capture device
14 image storage device

Figure 2 — Example of microscope system

If the initially obtained image does not sufficiently differentiate or characterize features, one should consider whether the microscope or system settings should be readjusted for increased image quality, the sample preparation process is sufficient, or the approach is otherwise not fit for purpose.

7.2.2 Light source

Light source should have an appropriate wavelength range, depending on the choice of the observation method and characteristics of the cell object(s). Light source should have sufficient intensity to obtain the necessary contrast (Figure E.1).

NOTE The term "light intensity" is used as a general term for the strength of light. It can be used to express absolute strength or relative strength of light. An appropriate unit needs to be used in order to express the strength of a particular light.

In particular, for fluorescence observation, a light source which produces sufficient fluorescence intensity should be selected in consideration of the following viewpoints;

a) wavelength range appropriate for the excitation of the fluorescent substance,

b) light intensity that provides sufficient excitation.

NOTE A narrow bandwidth of the target wavelength range can lead to insufficient excitation and cause low fluorescence if the intensity of the light source is attenuated.

The light source should provide sufficient contrast in the captured ROI and be appropriately adjusted so that phototoxicity and fading are not a problem for the cells.

The light source should be stable such that changes in cell morphological descriptor value(s) due to light fluctuations are negligible.

The wavelength and intensity of the light source used in the observations can affect cell properties. As a result, there are cases where the illuminated cells are not suitable for subsequent culture.

7.2.3 Objective lens and condenser lens

Optical resolution, depth of focus and aberrations of the objective and condenser lenses should be within a particular range according to the purpose of the image capture. The appropriate NAs should be selected according to the required resolution. In selecting an objective lens, the information on the surface of the objective lens is helpful. Marking of objective lenses is described in ISO 8578[1].

NOTE Optical resolution and aberrations are affected by material and thickness of the vessel entering the optical path.

In the case of an immersion type of objective lens, the refractive index of immersion liquid should be selected according to its optical design.

Specifications of the objective lens and the condenser lens shall be documented.

7.2.4 Components in optical path

Optical components that form optical path of the microscope system should be compatible to the wavelength of the light(s). The light means the illumination light and the light emitted from the cells.

NOTE 1 Other optical components include lens, mirrors and filters.

NOTE 2 Mismatch between the wavelength of light used and the properties of optical component causes decrease in transmittance of light through optical components.

For fluorescence observation, optical components, e.g., excitation filter, dichroic mirror, and fluorescence filter, should be selected so that the fluorescence light can be distinguished from the excitation light.

Specifications of used optical components shall be documented. The optical components can include but are not limited to:

a) spectral characteristics of the excitation filter, fluorescence filter and dichroic mirror;

b) objective lens;

c) condenser characteristics (e.g. numerical aperture, use of phase contrast filters);

d) model of microscope stand;

e) image capture device.

NOTE 3 Application of optical components such as filters and dichroic mirror are utilized for the selection of excitation light and the selective detection of fluorescence lights.

When observing simultaneously a TOI that has been labelled by multiple fluorescence dyes, the image capture devices should be properly configured to detect each fluorescent signal separately.

7.2.5 Image capture device

Captured image needs sufficient spatial resolution. The image capture device should have the pixel resolution necessary to achieve it.

The spatial resolution of the image shall be documented.

NOTE 1 The spatial resolution depends on a combination of optical resolution and pixel resolution.

The sensitivity and the dynamic range, of the image capture devices should be both considered to capture the image of the TOI in sufficient contrast.

NOTE 2 The sensitivity of the image capture devices is affected by the wavelength profile of the incident light. Some image capture devices use nonlinear sensitivity curves to increase dynamic range.

If the sensitivity of the image capture devices is insufficient, a higher intensity light source is needed. In such a case, it can cause phototoxicity or a photochemical change.

7.2.6 Image data

Image data shall have sufficient pixel resolution and bit depth sufficient for intended segmentation of the TOI. In the case when image data is compressed, attention should be taken to maintain sufficient data for segmentation. Any image compression processes shall be documented.

Image data should be accompanied with the meta data (e.g., image size, pixel resolution, bit depth, compression type, data format) to maintain reusability of the image.[14] Additional details are given in Clause 11.

7.2.7 Environmental conditions

During observation, it is necessary to minimize environmental change which can cause alternation in cell functions. The factors that can affect cell functions include, but are not limited to, composition of culture medium, temperature, humidity, carbon dioxide concentration and oxygen concentration.

The environmental condition shall be documented.

7.2.8 Observing position

The FOV shall be adjusted so that the TOI is captured as intended.

Focus shall be adjusted so that the TOI is captured as intended.

The image should be captured at the area where aberrations and distortions caused by the objective lens are negligible with respect to the intended purpose.

NOTE 1 Peripheral area of the image field tends to have larger aberrations compare to the centre of image field.

NOTE 2 The transmitted light observation of cells in the area adjacent to vessel wall can have image quality problems due to the meniscus effect of the culture medium.

7.2.9 Image capturing conditions

Light source intensity, camera gain and exposure time should be adjusted so that the dynamic range of the image capture device and the captured image contrast are both sufficient. Intensity values of pixels included in the TOI should not be saturated. The saturation of pixel intensity can cause loss of morphological feature information.

NOTE 1 In digital images, each pixel has an associated intensity value. “Brightness” refers to the overall visual perception of lightness or darkness in an image, and it is influenced by the collective intensity values of all the pixels. A “bright image” has higher average intensity values across its pixels, making it appear lighter.

NOTE 2 Automatic gain control can introduce incomparability between targets of interest. The use of dynamic camera gain settings is not appropriate if quantitative comparison of images is performed.

The signal to background noise ratio should be sufficient for the intended purpose.

NOTE 3 Excessive illumination intensity can cause phototoxicity or a photochemical change resulting in a change of the captured image.

7.3 Execution of image capture

7.3.1 Single image capture

Image capture shall be performed under the conditions established as described in 7.2.

7.3.2 Multiple image capture

Multiple images can be captured sequentially on the X-axis, Y-axis, Z-axis, and time-axis. Multiple image capture includes, but is not limited to, tile capture, Z-stack capture, high-speed capture and time-lapse capture[15].

NOTE 1 When capturing an image of a large FOV with high resolution, tiling and stitching technique can be used.

Image acquisition setup for tile capture should take into account the accuracy of the spatial positioning of X-Y position.

Image acquisition setup for Z-stack capture should take into account the size and number of Z-steps.

Image acquisition setup for time series capture should take into account the time increment and total acquisition time.

The temporal resolution of the imaging should be high enough to capture the changes of the TOI over time.

NOTE 2 The temporal resolution of the image capturing device for high-speed imaging is generally chosen to be shorter than half of the temporal resolution required for cell observations.

NOTE 3 The temporal resolution is determined by exposure time, external trigger delay, stable operation throughout the observation period and the transfer speed of image data.

The stage of microscope should have sufficient stability over the time and repeatability of positioning in plane and vertical coordinates to achieve multiple image capturing. The positioning error should be considered for the uncertainty of cell morphological descriptors.

8 Segmentation of the TOI

The TOI should be identified from the acquired image in order to evaluate its morphological feature. It should be taken into account that the TOI can be changed by the bioprocess.

It should be confirmed that each segmented object corresponds to a TOI in view of the intended purpose. Both images, before and after segmentation, should be recorded, so that the segmentation process can be reviewed.

Segmented object should meet at least one of the following.

a) Each pixel composing segmented object is classified.

b) The contour of each segmented object is represented by the coordinate points.

When images are analysed by methods which do not utilize the TOI segmentation, the method should be documented.

NOTE Application of artificial intelligence (AI) tools is one of the methods which does not necessarily utilize the TOI segmentation.

9 Quantification

9.1 General

Quantitative description of cell morphology is established through quantification of cell shape, size, texture, or multiple of these of a segmented object by applying appropriate formula.

To describe morphological characteristics of a segmented object, cell morphological descriptors are often used in combination. Examples of such cell morphological descriptors include area, major axis length, minor axis length, circularity, and convexity.

Lists of commonly applied cell morphological descriptors and their formulae are provided in Table C.1 and Table D.1.

NOTE A wide range of texture representation has been developed over the years[16]. There is no formal route to identify the optimal texture representation for a given use case.

9.2 Procedure to quantify morphological features of segmented object

One or more cell morphological descriptors which are suitable for the evaluation purpose shall be studied.

Morphological features of segmented objects should be quantified by evaluating the cell morphological descriptors. Requirements for quantification include the following:

a) Name, definition and formula of the cell morphological descriptor applied for calculation shall be clarified and shall be recorded.

b) In the case when introducing a novel cell morphological descriptor that is not commonly used, the cell morphological descriptor shall be presented along with its definition and formula.

c) When a morphological feature of the segmented object is to be described without using cell morphological descriptors, an algorithm to describe such morphological feature shall be provided. The algorithm can be explained by means of formula, illustrations, or sentences. Scientific references can also be taken into account.

10 Qualification of Measurement

Measurement should be qualified by confirmation of its achievement in accordance with the pre-determined procedures.

Result of qualification shall be documented.

NOTE The documentation can also contain other relevant metadata of the bio-measurement as discussed in the REMBI guidelines[11].

11 Reporting

11.1 General

Appropriate description of the experimental system in the report of cell morphometry is critical for the obtained data to be meaningful.

The report for cellular morphological analysis shall contain sufficient information to allow independent assessment of the cellular morphological analysis, in view of sample preparation, microscopic observation method, image processing/image analysis, and morphological features.

NOTE 1 The report can help to enable reproducibility and interpretation of the morphometric results.

NOTE 2 Several resources are available that can help to identify critical reporting elements based on the application and type of microscopy used for the morphometric analysis[11,12,17].

The specific elements within the report regarding 11.2 through 11.5 can vary depending on the intended purpose of the morphological assessment and the nature of the experimental design.

11.2 Reporting of sample properties and sample preparation

The report shall contain information on the sample used for the morphometric analysis.

The report should include information on sample properties and sample preparation including but not limited to the following when applicable:

a) cell type and origin;

b) observation vessel (7.1.3.3);

c) fixation procedures and reagents (7.1.3.2);

d) labelling procedures and reagents including (7.1.3.2):

   1) excitation characteristics;

   2) emission characteristics.

NOTE Numbers in parentheses after listed items in 11.2 through 11.4 represent relevant clauses of this document.

11.3 Reporting of the microscopic observation method

The report shall contain information on the microscopic observation method used for the morphometric analysis.

Reporting elements related to the microscopic observation method should include but are not limited to the following when applicable:

a) microscope model and manufacturer;

b) contrast-enhancing techniques (7.1.1, 7.1.2);

c) light source(s) (7.2.2);

d) optical components including (7.2.3, 7.2.4):

   1) filter(s);

   2) dichroic mirror(s);

   3) lens(es);

4) objective lens(es);

e) image capture device (7.2.5);

f) environmental control approaches (i.e., when conducting live cell imaging, composition of culture medium, temperature, humidity, carbon dioxide concentration and oxygen concentration) (7.2.7);

g) image acquisition set-up (7.3).

h) others (if applicable, e.g., resolution enhancement technique)

NOTE 1 The image capture device is sometimes referred to as the detection system. An instrument can have more than one detection systems of different types, such as a photomultiplier or a camera[12].

NOTE 2 A comprehensive review of resolution enhancement techniques in microscopy is available [18].

11.4 Reporting of the image data, image pre-processing, and image analysis for morphometric analysis

The report shall contain information on the image data, image processing, and image analysis used for the morphometric analysis.

Reporting elements related to image data, image processing, and image analysis should include but are not limited to the following when applicable:

a) image data information including (7.2.6):

1) effective pixel size in the image in micrometres (i.e., pixel to micrometre ratio of the image);

2) bit-depth of the image data (e.g., 8-bit, 16-bit, RGB (red, green, blue));

3) size of the images in pixels in x and y directions;

4) image compression procedures used prior to morphometric analysis;

5) number of channels

NOTE Multi-channel acquisitions are frequently performed by automated microscopy. It means that the same spatial location is imaged with either multiple different excitation/emission wavelengths, multiple transmitted light contrast modes, or both.

b) image pre-processing information including (7.2.8, 7.2.9, 7.3.2):

1) image stitching for tiled acquisitions;

2) correction for non-uniform illumination, non-uniform background signal, or both across the field of view;

3) intensity normalization;

4) others (if applicable, e.g., background subtraction)

c) Image analysis information including the following (see Clause 8):

1) algorithm(s) for segmentation of the TOI;

2) object separation and selection processes (e.g., watershed, erosion, dilation, size exclusion etc.).

For morphometric analysis, uncompressed image data should be used instead of compressed image data.

NOTE A comprehensive review of open-source image analysis tools for cells including algorithms is available[19].

11.5 Reporting of the morphological features for morphometric analysis

The report shall contain information on the morphological features for morphometric analysis including the following (see Clause 9):

a) name of cell morphological descriptor;

b) definition of the cell morphological descriptor;

c) (if applicable) description of algorithm for each morphological feature of the segmented TOI including (e.g., formula, illustrations, or sentences).

NOTE 1 Morphological features can be reported by referring to the cell morphological descriptors and their numbers in this document.

NOTE 2 For cell morphological descriptors listed in Table C.1 and Table D.1, morphological features can be reported as the cell morphological descriptor and its number while referencing this document.

NOTE 3 Inclusion of item b), item c), or both is described in 9.2 c).

Annex A
(informative)

Check sheet regarding selection of contrast-enhancing techniques for optical microscopy

NOTE Table A.1 includes examples of the technique for contrast-enhancement, which can be selected based on the intended use.

Table A.1 — Check sheet regarding selection of contrast-enhancing techniques for optical microscopy

<table>
<tr><th>Item of consideration</th><th>Brightfield</th><th>Phase-contrast</th><th>DIC</th><th>Darkfield</th><th>Fluorescence</th></tr>
<tr><td rowspan="2">Cell properties to be observed -- Adherence and suspension</td><td></td><td colspan="2">An adhered cell tends to spread in, thin layers. This makes it difficult to perform a bright field observation of a transparent cell. Methods that utilize difference in refractive indices, such as phase contrast or differential interference can be used.</td><td></td><td rowspan="2"></td></tr>
<tr><td colspan="4">Contrast will increase when the cell becomes spherical. This can be experienced in floating cells, which are being cultured in a liquid medium, or cells that are being cultured in a semisolid medium.</td></tr>
<tr><td rowspan="2">Cell properties to be observed -- Stack and stratification</td><td colspan="4">Most transmission-type methods are not adequate for observation of deep 3D structures because the illumination light is scattered, and less light is transmitted through the sample. Using long wavelength light for observation can reduce scattering.</td><td>Imaging of deep 3D structures can be supported by light sheet microscopy</td></tr>
<tr><td>Depending on sample properties confocal reflection microscopy can offer improved image quality</td><td>When single-layer or multi-layer cells have significant variations in cell-layer thickness and height, a "halo" image effect appears around the cells. This effect lowers image quality in phase-contrast observation.</td><td></td><td>With the fluorescence observation method, vertical light from the surrounding of the TOI cell(s) can lower image quality. This problem can be reduced using confocal optics or 2-photon microscopy.</td></tr>
<tr><td rowspan="3">Labelling, dyeing, and chemical treatments</td><td colspan="5">Elapsed time and culture environment can affect cell activity and sensitization during observation or cause a photochemical change of the stained material.</td></tr>
<tr><td colspan="5">Fluorescent labelling, immunostaining, or other chemical treatments can alter cell membrane and nuclear properties, as well as other cell characteristics. As a result, the morphological characteristics of the treated cells can be different from those of untreated living cells.</td></tr>
<tr><td colspan="4"></td><td>The wavelength and intensity of the light source used in the observations can affect cell properties.</td></tr>
<tr><td>Intracytoplasmic structures</td><td colspan="3">Existence of intracytoplasmic structures increases the cell contrast, therefore cells with sufficient amount of intracytoplasmic structures can be observed</td><td colspan="2"></td></tr>
<tr><td rowspan="2">Vessels</td><td colspan="2"></td><td>Vessels made of polymeric material are often not appropriate for differential interference microscopy because of birefringence.</td><td colspan="2"></td></tr>
<tr><td colspan="5">Working distance defined as the distance from a front lens element of the objective to a TOI when the TOI is in focus, depends on the optical design of the objective, such as magnification and NA. It is important to use a vessel whose thickness entering the optical path is less than the working distance.</td></tr>
</table>

Annex B
(informative)

Check sheet regarding selection of microscope system

Table B.1 — Check sheet regarding selection of microscope system

Items	Check
General (7.2.1)	
The microscope system is maintained properly[13].	☐
Light source (7.2.2)	
Light source has an appropriate wavelength range, depending on the choice of the observation method and characteristics of the cell object(s)	☐
Light source has sufficient intensity to obtain the necessary contrast	☐
Light source which produces sufficient fluorescence intensity is selected in consideration of the following viewpoints; a) wavelength range appropriate for the excitation of the fluorescent substance; b) light intensity that provides sufficient energy for the excitation	☐
Light source is stable	☐
Light source is set to an intensity that does not cause phototoxicity or discoloration (related to 7.2.5)	☐
Objective lens (7.2.3)	
Optical resolution, depth of focus, and aberration of the objective lens is within a particular range according to the purpose of image capture	☐
The appropriate NA is selected according to the required resolution	☐
Microscope system has an objective lens with NA and illumination wavelength appropriate for the required resolution of the object to be observed	☐
(In the case of an immersion type of objective lens) The refractive index of immersion liquid is selected according to its optical design, and the immersion liquid is compatible with slide material	☐
Components in optical path (7.2.4)	
Optical components that form optical path of the microscope system is compatible to the wavelength of the light(s)	☐
Optical components are selected so that the fluorescence light can be distinguished from the excitation light	☐
(When observing simultaneously TOI that has been labelled by multiple fluorescence dyes) The image capture devices is properly configured to detect each fluorescent signal separately	☐
Image capture device (7.2.5)	
Image capture device has the pixel resolution necessary to achieve sufficient spatial resolution	☐
The sensitivity and dynamic range, of the image capture devices have both considered to capture the image of the TOI in sufficient contrast	☐

Annex C
(informative)

List of cell morphological descriptors, definitions and formulae for cell shape, size

Table C.1 — List of morphological descriptors, definitions and formulae

No.	Cell morphological descriptor	#	Definition	Formula	Notes
S-1	**Area**		The actual number of pixels in the region	A_{area} N/A (count pixels)	For per-pixel measurements (length, area, etc.), pre-define the size per pixel
S-2	**Perimeter**	1	The total number of pixels around the boundary of each region in the image.	N/A	
		2	Cauchy-Crofton formula *Supplementary explanation: Calculated from the number I_θ where parallel lines with an angle θ and an interval d_L straddle the boundary.	$P = \frac{\pi \cdot d_L}{N} \sum_{i=1}^{N} I_\theta (d_L) \mid \theta = \pi(i-1)/N$	The estimate for the perimeter P is calculated from the number of intercepts I_θ of a series of parallel lines with spacing d_L and the target region by exploring N different directions θ.

Table C.1 *(continued)*

No.	Cell morphological descriptor	#	Definition	Formula	Notes
S-3	**Major Axis Length**		Long axis when approximated by the Legendre ellipse (defined by the moment of inertia = σ_{xx}, σ_{yy}, σ_{xy})	Max Length (of Legendre ellipse): $l_{max} = 4\sqrt{\alpha+\beta}$ Definition of intermediate terms $\alpha = \frac{1}{2}(\sigma_{xx}+\sigma_{yy})$ $\beta = \sqrt{\alpha^2 - \sigma_{xx}\sigma_{yy}+\sigma_{xy}}$ Determination of the moments of inertia of the shape coordinates. $\sigma_{xx} = \frac{1}{n}\Sigma(x_i-\bar{x})^2$ $\sigma_{yy} = \frac{1}{n}\Sigma(y_i-\bar{y})^2$ $\sigma_{xy} = \frac{1}{n}\Sigma(y_i-\bar{y})(x_i-\bar{x})$ $\bar{x} = \frac{1}{n}\Sigma x_i$ $\bar{y} = \frac{1}{n}\Sigma y_i$ where x_i is the i-th pixel coordinate x, y_i is the i-th pixel coordinate y, n is the number of object pixels	

Table C.1 *(continued)*

No.	Cell morphological descriptor	#	Definition	Formula	Notes
S-4	**Minor Axis Length**		Short axis when approximated by the Legendre ellipse (defined by the moment of inertia = σ_{xx}, σ_{yy}, σ_{xy})	Min Length (of Legendre ellipse): $l_{\min} = 4\sqrt{\alpha - \beta}$ Definition of intermediate terms Determination of the moments of inertia of the shape coordinates. $\alpha = \frac{1}{2}(\sigma_{xx} + \sigma_{yy})$ $\beta = \sqrt{\alpha^2 - \sigma_{xx}\sigma_{yy} + \sigma_{xy}}$ $\sigma_{xx} = \frac{1}{n}\Sigma(x_i - \bar{x})^2$ $\sigma_{yy} = \frac{1}{n}\Sigma(y_i - \bar{y})^2$ $\sigma_{xy} = \frac{1}{n}\Sigma(y_i - \bar{y})(x_i - \bar{x})$ where x_i is the i-th pixel coordinate x, y_i is the i-th pixel coordinate y, n is the number of object pixels	
S-5	**Feret maximum**		The Feret diameter is the distance between two parallel lines tangent on either side of the object. The maximum Feret diameters are the largest possible diameters, rotating the distance between two parallel lines along all possible angles.	$D(F_{\max})$ N/A (Count from the image)	
S-6	**Feret minimum**		The Feret diameter is the distance between two parallel lines tangent on either side of the object. The minimum Feret diameters are the smallest possible diameters, rotating the distance between two parallel lines along all possible angles.	$D(F_{\min})$ N/A (Count from the image)	
S-7	**Aspect ratio**		Length ratio of the axes of the Legendre ellipse	$R_a = \frac{l\max}{l\min}$	

Table C.1 *(continued)*

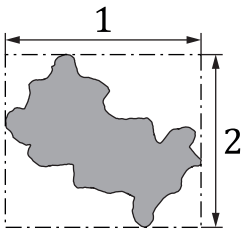
No.	Cell morphological descriptor	#	Definition	Formula	Notes
S-8	**Elongation**	1	Ratio of the shortest Feret diameter to the longest Feret diameter	$E_{elong} = \frac{D(F_{min})}{D(F_{max})}$	See S-5 and S-6.
		2		$E_{elong} = \frac{D(F_{max})}{D(F_{min})}$	
S-9	**Irregularity**		Relationship between the diameter of the maximum inscribed circle: d_{imax} and that of the minimum circumscribed circle: d_{cmin}	$R_{irr} = \frac{d_{imax}}{d_{cmin}}$	
S-10	**Box ratio**		Ratio of the Feret box area to the projected area	$B_{ratio} = \frac{A}{A_{box}}$ $A_{box} = D(F_{min}) \cdot D(F_{per})$ $D(F_{per})$ is the Feret diameter perpendicular to the Feret minimum.	
S-11	**Extent**  **Key** (1) Feret X (2) Feret Y	1	The proportion of the pixels in the bounding box that are also in the region. Computed as the Area divided by the area of the bounding box.	$E_{ext} = \frac{A}{B_{area}}$ $B_{area} = D(F_X) \cdot D(F_Y)$	
		2		$E_{ext} = \frac{A}{D(F_{max}) \cdot D(F_{min})}$	

Table C.1 *(continued)*

No.	Cell morphological descriptor	#	Definition	Formula	Notes
S-12	**Compactness** **(Roundness)**	1	Ratio of actual perimeter to perimeter when assuming a circle	$C_{\text{compact}} = \frac{P^2}{4 \cdot \mathrm{A} \cdot \pi}$ NOTE There are cases where a specific PERIMETER other than those described in S-2 need to be defined for determination of COMPACTNESS	Perimeter (S-2) used in the formula also has multiple choices for its definition/ formula, therefore it is necessary to indicate which set of definition/ formula was used. **sample description:** *compactness: S-11/formula2 (S-2/formula2 is used as perimeter in the formula)*
		2	(See the formula)	$C_{\text{compact}} = \frac{4 \cdot A_{\text{area}} \cdot \pi}{l(P_{\text{convexhull}})^2}$ where $P_{\text{convexhull}}$ is the perimeter length of the smallest convex shape enclosing a given shape.	
		3	Degree to which the particle (or its projection area) is similar to a circle, considering the overall		Basically, "Roundness" should not be used as a name, however when describing "Roundness", it should be written together with (=Compactness) and which of the above definitions and formulas is used.
S-13	**Circularity**	1	With a value of 1:0 indicating a perfect circle. As the value approaches 0:0, it indicates an increasingly elongated shape. There are cases where values are not be valid for very small particles.	$C_{\text{circularity}} = \frac{4 \cdot A_{\text{area}} \cdot \pi}{P^2}$	Perimeter (S-2) used in the formula also has multiple choices for its definition/ formula, therefore it is necessary to indicate which set of definition/ formula was used. **sample description:**
		2	(See the formula)	$C_{\text{circularity}} = \frac{A_{\text{area}}}{\pi \cdot (R_{\text{min}})^2}$ R_{min} is the radius of the minimum circumscribed circle *supplementary explanation: Area of object relative to area of circumscribed circle	*Circularity: S-13/formula2 (S-2/formula2 is used as perimeter in the formula)* In physical metrology, circularity and roundness are sometimes used synonymously.
		3	Degree to which the object region is similar to a circle, considering the smoothness of the perimeter.	$C_{\text{circularity}} = \frac{A_{\text{area}}}{\pi \cdot (R_{\text{min}})^2}$	

Table C.1 *(continued)*

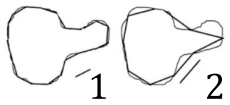
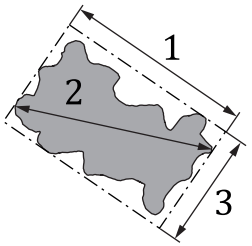
No.	Cell morphological descriptor	#	Definition	Formula	Notes
S-14	**Solidity**		The proportion of the pixels in the convex hull that are also in the object, i.e., Object Area/Convex Hull Area		
S-15	**Convexity**	1	As an alias for solidity; "Also known as convexity"	$C_{convex} = \frac{A_{area}}{A_{convexhull}}$ $A_{convexhull}$: Area of the smallest convex shape enclosing a given shape.	Perimeter (S-2) used in the Formula 2 also has multiple choices for its definition/formula, therefore it is necessary to indicate which set of definition/ formula was used. **sample description:** *Convexity: S-15/formula2 (S-2/formula2 is used as perimeter in the formula)*
		2	(See the formula)	$C_{convex} = \frac{l(P_{convexhull})}{l(P)}$ where $P_{convexhull}$ is the perimeter of the smallest convex shape enclosing a given shape.	
S-16	**Fractal dimension**  **Key** (1) λ (2)2 λ		The relationship between the length of the perimeter P(λ) and the length λ of the step is linear on a log-log plot, known as a Richardson plot. The data are first normalized by dividing by the maximum Feret diameter. The upper limit for the step size is given by: $\lambda = 0{,}3x_{Fmax}$	The formula of the straight line is: $\log P(\lambda) = (1 - D_F)\log\lambda + \log b$ where D_F is the fractal dimension	Reference [2].
S-17	**Area Box**  **Key** (1) Feret diameter perpendicular to the Feret minimum		(See the formula)	$A_{box} = \frac{D(F_{min})}{D(F_{per})}$ where $D(F_{per})$ is the Feret diameter perpendicular to Feret minimum"	

Table C.1 *(continued)*

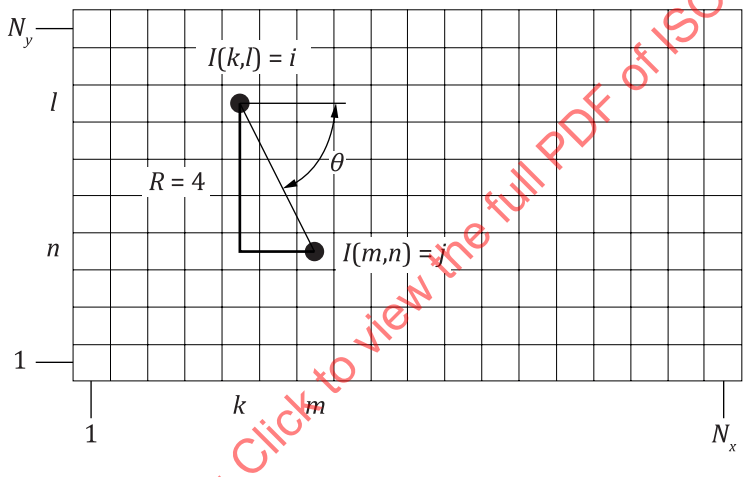
No.	Cell morphological descriptor	#	Definition	Formula	Notes
	(2) Feret maximum (3) Feret minimum				

Annex D
(informative)

List of cell morphological descriptors, definitions and formulae for cell texture

Textural features of an image can be derived from analysis of the GLCM (Gray Level Co-occurrence Matrix). The GCLM is a tabulation of how often different combinations of pixel brightness values (grey levels) occur in an image. GLCM is a matrix whose component (i,j) is the probability that the point separated by a certain displacement R and angular direction θ from the point of pixel brightness values = i is the point that the pixel brightness values = j. The distance between two pixel-positions $\langle k,l\rangle$ and $\langle m,n\rangle$ *is* measured as given in Formula (D.1) and a schematic image is given in Figure D.1:

$$R(\langle k,\ l\rangle,\ \langle m,\ n\rangle)=\max(|k-m|,|l-n|) \tag{D.1}$$



Key

N_x	horizontal image size in number of pixels
N_y	vertical image size in number of pixels
k, m	horizontal positions in number of pixels
n, l	vertical positions in number of pixels
$I(k, l)$	grey scale value of pixel at position (k, l)
$I(m, n)$	grey scale value of pixel at position (m, n)
R	distance between pixel positions defined in Formula (D.1)
θ	angular direction from pixel position (k, l) to position (m, n)

Figure D.1 — Schematic digital image

Formulae are given in the following for analysis of neighbouring pixels (i.e. R =1) for which the direction θ can take the value 0°, 45°, 90° or 135°. The image is assumed to have a size of N_x horizontal pixels and N_y pixels in vertical direction; and to have N_g grey scale values. $I(k,l)$ is the index of the grey scale at pixel position (k,l).

GCLM based image analysis can use a selection of any of the five following normalised frequencies $p\ (i, j)$:

$$p(i,j,0°)=\frac{1}{2\times(N_x-1)\times N_y}\times\sum_{k=1}^{N_x-1}\sum_{l=1}^{N_y}\left(\delta_{i,I(k,l)}\times\delta_{j,I(k+1,l)}+\delta_{j,I(k,l)}\times\delta_{i,I(k+1,l)}\right) \quad \text{(D.2)}$$

$$p(i,j,45°)=\frac{1}{2\times(N_x-1)\times(N_y-1)}\times\sum_{k=1}^{N_x-1}\sum_{l=2}^{N_y}\left(\delta_{i,I(k,l)}\times\delta_{j,I(k+1,l-1)}+\delta_{j,I(k,l)}\times\delta_{i,I(k+1,l-1)}\right) \quad \text{(D.3)}$$

$$p(i,j,90°)=\frac{1}{2\times N_x\times(N_y-1)}\times\sum_{k=1}^{N_x}\sum_{l=1}^{N_y-1}\left(\delta_{i,I(k,l)}\times\delta_{j,I(k,l+1)}+\delta_{j,I(k,l)}\times\delta_{i,I(k,l+1)}\right) \quad \text{(D.4)}$$

$$p(i,j,135°)=\frac{1}{2\times(N_x-1)\times(N_y-1)}\times\sum_{k=1}^{N_x-1}\sum_{l=1}^{N_y-1}\left(\delta_{i,I(k,l)}\times\delta_{j,I(k+1,l+1)}+\delta_{j,I(k,l)}\times\delta_{i,I(k+1,l+1)}\right) \quad \text{(D.5)}$$

$$p_{ave}(i,j)=(p(i,j,0°)+p(i,j,45°)+p(i,j,90°)+p(i,j,135°))/4 \quad \text{(D.6)}$$

where δ denotes the Kronecker delta.

Table D.1 — List of cell morphological descriptors, definitions and formulae for cell texture

No.	Cell morphological descriptor	Definition	Formula	Notes
T-1	Angular second moment	Measure of image homogeneity. A higher value of this feature indicates that the intensity varies less in an image. Has a value of 1 for a uniform image.	$f_1 = \sum_i \sum_j \{p(i,j)\}^2$	T1 to T14 quote from Reference [21]. T1 to T14 are based on the following formulae in addition to Reference [21]:
T-2	Contrast	Measure of local variation in an image, with 0 for a uniform image and a high value indicating a high degree of local variation	$f_2 = \sum_{n=0}^{N_g-1} n^2 \left\{ \sum_{i=1}^{N_g} \sum_{\substack{j=1 \\ \lvert i-j \rvert = n}}^{N_g} p(i,j) \right\}$	Notation $p_x(i)$ is the i th entry in the marginal-probability matrix obtained by summing the rows of $p(i,j)$, $p_y(j)$ is the j th entry in the marginal-probability matrix obtained by summing the columns of $p(i,j)$, N_g is the number of distinct gray levels in the quantized image.

Table D.1 *(continued)*

No.	Cell morphological descriptor	Definition	Formula	Notes
T-3	Correlation	Measure of linear dependency of intensity values in an image. For an image with large areas of similar intensities, correlation is much higher than for an image with noisier, uncorrelated intensities. Has a value of 1 or -1 for a perfectly positively or negatively correlated image, respectively	$f_3 = \frac{\sum_i \sum_j (ij) p(i,j) - \mu_x \mu_y}{\sigma_x \sigma_y}$	$p_x(i) = \sum_{j=1}^{N_g} p(i,j)$ $p_y(j) = \sum_{i=1}^{N_g} p(i,j)$ $p_{x+y}(k) = \sum_{i=1}^{N_g} \sum_{\substack{j=1 \\ i+j=k}}^{N_g} p(i,j)$ $k = 2,3,\cdots, 2N_g$ $p_{x-y}(k) = \sum_{i=1}^{N_g} \sum_{\substack{j=1 \\ \lvert i-j\rvert=k}}^{N_g} p(i,j)$ $k = 0,1,\cdots, N_g - 1$
T-4	Variance	Measure of the variation of image intensity values. For an image with uniform intensity, the texture variance would be zero.	$f_4 = \sum_i \sum_j (i-\mu)^2 p(i,j)$	
T-5	Inverse difference moment	Another feature to represent image contrast. Has a low value for inhomogeneous images, and a relatively higher value for homogeneous images.	$f_5 = \sum_i \sum_j \frac{1}{1+(i-j)^2} p(i,j)$	
T-6	Sum average	The average of the normalized gray-scale image in the spatial domain.	$f_6 = \sum_{i=2}^{2N_g} i p_{x+y}(i)$	
T-7	Sum variance	The variance of the normalized gray-scale image in the spatial domain	$f_7 = \sum_{i=2}^{2N_g} (i - f_8)^2 p_{x+y}(i)$	
T-8	Sum entropy	A measure of randomness within an image.	$f_8 = \sum_{i=2}^{2N_g} p_{x+y}(i) \log\{p_{x+y}(i)\}$	
T-9	Entropy	An indication of the complexity within an image. A complex image produces a high entropy value.	$f_9 = -\sum_i \sum_j p(i,j) \log\{p(i,j)\}$	
T-10	Difference variance	The image variation in a normalized co-occurrence matrix.	$f_{10} = \text{variance of } p_{x-y}$ $= \sum_{k=0}^{n-1} \left\{ k - \sum_{k-0}^{n-1} k p_{x-y}(k) \right\}^2 P p_{x-y}(k)$	
T-11	Difference entropy	Another indication of the amount of randomness in an image.	$f_{11} = -\sum_{i=0}^{N_g - 1} p_{x-y}(i) \log\{p_{x-y}(i)\}$	

Table D.1 *(continued)*

No.	Cell morphological descriptor	Definition	Formula	Notes
T-12	Information measures of correlation 1	A measure of the total amount of information contained within a region of pixels derived from the recurring spatial relationship between specific intensity values.	$f_{12} = \frac{HXY - HXY1}{\max\{HX, HY\}}$ $HXY = -\sum_i \sum_j p(i,j)\log(p(i,j))$ where HX and HY are entropies of p_x and p_y and $HXY1 = -\sum_i \sum_j p(i,j)\log\{p_x(i)p_y(j)\}$ $HXY2 = -\sum_i \sum_j p_x(i)p_y(j)\log\{p_x(i)p_y(j)\}$	
T-13	Information measures of correlation 2	An additional measure of the total amount of information contained within a region of pixels derived from the recurring spatial relationship between specific intensity values. It is a complementary value to InfoMeas1 and is on a different scale.	$f_{13} = (1 - \exp[-2.0(HXY2 - HXY)])^{1/2}$ $HXY = -\sum_i \sum_j p(i,j)\log\{p(i,j)\}$ where HX and HY are entropies of p_x and p_y and $HXY1 = -\sum_i \sum_j p(i,j)\log\{p_x(i)p_y(j)\}$ $HXY2 = -\sum_i \sum_j p_x(i)p_y(j)\log\{p_x(i)p_y(j)\}$	
T-14	Maximal correlation coefficient	N/A	f_{14} $= (\text{Second largest eigenvalue of Q})^{1/2}$ where $Q(i,j) = \sum_k \frac{p(i,k)p(j,k)}{p_x(i)p_y(k)}$	

Annex E
(informative)

Points to consider when acquiring phase-contrast images of cells suitable for image analysis[1)]

E.1 Setting the analysis target/objectives

E.1.1 General

A common use case of image analysis is for routine evaluation of the characteristics of cells.

Image analysis can in some cases be performed on raw data, but often when the image is checked again, there are variations in conditions such as brightness, imaging position, focus, and image resolution, which can frustrate the image analysis algorithm.

When it is difficult to develop an algorithm, it can be more efficient to simply re-take the image and use it for analysis instead.

Conversely, there can also be cases where even if the acquisition settings at the time of imaging are relaxed (lower image resolution than previous, imaging position not strictly controlled, etc.), it does not become problematic given the analysis objectives.

That is, the following two points are important for performing image analysis:

a) Define the target (i.e., what is the cell state? Which part of the cell is being observed?) and objective (i.e., what needs to be clarified?) of the analysis to be performed.

   — For example:

      1) Only the area occupied by cells in the field of view is of interest, so distinguishing cells from background is necessary.

      2) A count of the number of cells is desired, so recognition of each individual cell is necessary, but not morphological characterization.

      3) Analysis of the shape of each cell is desired, so it is necessary to accurately recognize the shape of the cell.

b) Obtain an image in which the analysis target has been clearly captured and can be accurately identified by eye.

   — Appropriate imaging conditions will vary according to the analysis target and objective. Typical acquisition settings are explained below in the context of the following analysis examples:

      1) Adherent cells (non-colony forming) (see E.1.2)

      2) Adherent cells (colony forming) (see E.1.3)

      3) Spheroids (see E.1.4)

1) The text, figures and tables of Annex E are derived from Reference [20] in agreement with the original copyright holder.

E.1.2 Adherent cells (non-colony forming)

Various evaluations are performed with adherent cells (non-colony forming). There are cases in which the objective of evaluation is to determine the cell count, or the cell confluency, which are generally used for routine evaluation of culture status. There are also cases in which the objective is to express morphological feature of specific cell type, e.g., neurite length of nerve cells.

NOTE 1 Table E.1 provides an example of imaging check-sheet for adherent cells (non-colony forming).

a) In the case of cell counting, analysis can be difficult because the difference in brightness between cells and the background is sometimes not significant, depending on the imaging conditions.

   — Obtain a cell image in which the contrast between cells and the background is as high as possible, the container is not noticeably dirty (including condensation), and the appearance does not differ greatly between cells. The acquisition of phase contrast images is sensitive to focus. If phase contrast is not applied, using brightfield as an example, de-focusing can be applied to increase contrast for cell counting at the expense of resolving other morphological features.

b) If adjacent cells are overlapping when cell morphology is to be quantified, analyzing each cell separately is difficult.

   — The effectiveness of identifying individual cells in images can be improved by reducing the cell density as much as possible so that adjacent cells do not overlap. For example, it is important for neurites to be in focus when neurite length is to be analyzed.

E.1.3 Adherent cells (colony forming)

Various evaluations are performed with adherent cells (colony forming). There are cases in which the objective of evaluation is to determine the cell count or the percent area occupied by cells, which are generally used for routine evaluation of culture status. There are also cases in which the objective is to express morphological feature of specific cell type, e.g., neurite length of nerve cells.

The area of a colony at a particular time point and the degree of differentiation/undifferentiation of cells in the colony are often analysed to evaluate the state of colony-forming adherent stem cells, such as induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs).

NOTE Table E.2 provides an example of an imaging check-sheet for adherent cells (colony forming).

a) When the area of small colonies is to be measured, or tracked just after seeding, it is important for the cell density to be low and the imaged field to be free of dead cells or debris.

   — Colony tracking is easier at lower cell densities. However, dead cells and debris can be detected as part of the colony under these conditions.

b) When assessing the differentiation/undifferentiation of a colony, the texture of the colony's surface is an important indicator.

   — The morphometric analysis can be improved if colony is in focus and the image is not oversaturated.

E.1.4 Spheroids

When evaluating the state of a spheroid, the area and roundness of the spheroid at a given time point are commonly analysed.

NOTE Table E.3 provides an example of imaging check-sheet for spheroids.

a) Spheroids are difficult samples to observe because they are thick, and therefore it is important to carefully control the focus to improve the repeatability of the morphological analysis.

   — Applicable measures to improve focusing on the surface include: