

# CalMorph

## User Manual

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## 0 What is CalMorph

CalMorph is a program that outputs a large amount of data on cell cycle phase, cell forms, etc., for individual cells, from a set of pictures of cell walls, cell nuclei, and actins.

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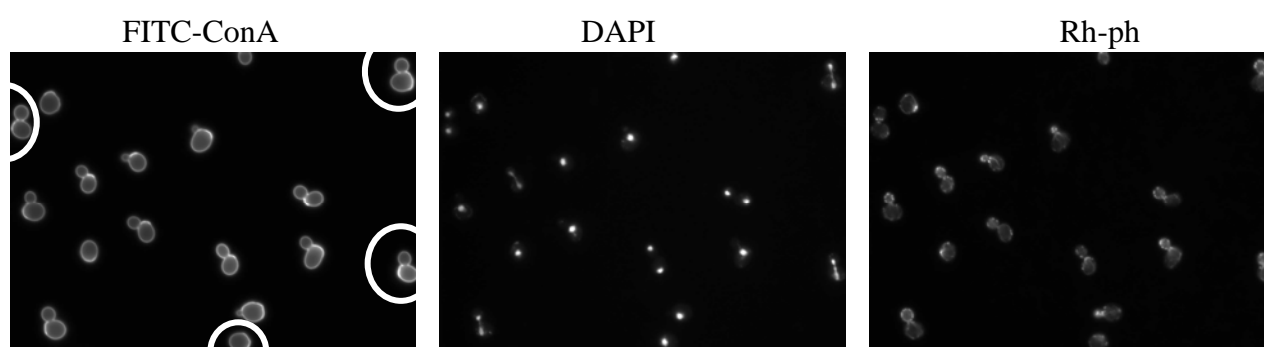
## 1 Preparation for microscopic image analysis

### 1.1 Image quality

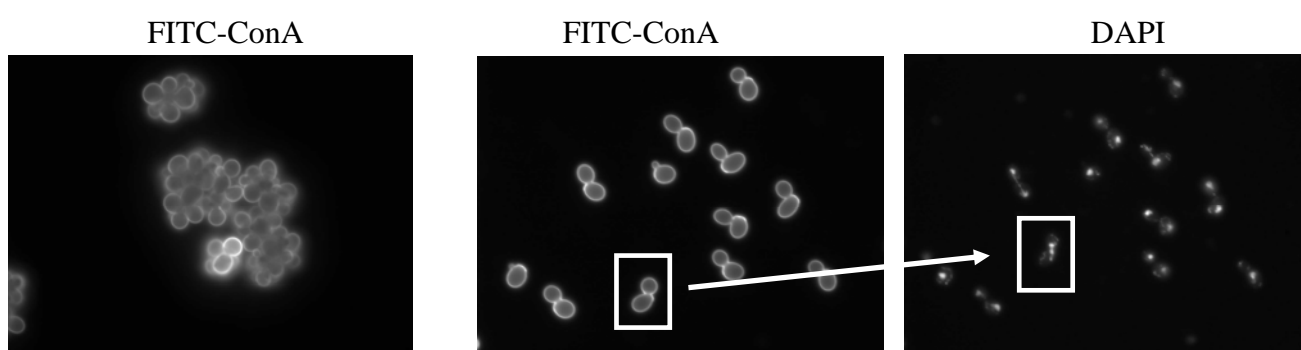
In the images, the cells should not be too close together, and the brightness of the cell wall images should be as uniform as possible (Good Images). It is difficult to extract data from cells at the edge of the image. For example, even in Good Images, CalMorph cannot obtain quantitative data from the cells surrounded by circles. If the cells are too close, or the brightness is excessively uneven, the program may fail to recognize individual cells, reducing the accuracy of the analysis. Incorrect data may result if more than two cells are stuck together (Bad Images, Left). In addition, it is highly probable that incorrect data may result for cells with shapes that differ greatly from an ellipse, because CalMorph approximates the shape of

mother cells and buds as elliptical. Furthermore, it may fail to process an image properly if there is too much noise, such as a coarse background.

Additionally, special care should be taken when obtaining cell images. Since the ratio of small cells is usually higher at the edge of a specimen, the images should be obtained from near the center of the coverslip. Small buds tend to be oriented vertically with respect to the coverslip. In order to avoid mistaken recognition, try to choose fields where small buds are visible at the in-focus position. Furthermore, cells sometimes move. When obtaining images from such a field, the cells may mistakenly be considered to lack a nucleus or actin (see Bad Images, the cell surrounded by a square). Let the slide sit, and acquire images from fields with



Good Images: The cells are evenly stained, and scattered throughout the image.



Bad Images: Sticky cells

A cell that moved during a filter change.

non-moving cells.

## **1.2 Image size and Image format**

CalMorph uses JPEG format images (extension jpg) with a 696×520 pixel size and grayscale (256 gradation). If the images to be analyzed are not in this format, they must be converted using software tools, such as ImageMagick, which can be downloaded from <http://www.imagemagick.org>.

## **1.3 Standardization of image magnification**

The magnification of a series of cell images to be compared should be the same, since the data obtained in the analysis depends on the size of the cells in the image.

## **1.4 Image file naming conventions (Serial numbering of image files)**

Group the image files to be analyzed and put them together in a folder with a name corresponding to the group name. Name the image files as follows:

- cell wall image: (group name)-C(number).jpg,
- cell nucleus image: (group name)-D(number).jpg,
- actin image: (group name)-A(number).jpg.

Moreover, assign the same number to cell wall, cell nucleus, and actin images belonging to the same set. For example, if the group name is his3, then name the cell wall images his3-C1.jpg, his3-C2.jpg..., the cell nucleus images his3-D1.jpg, his3-D2.jpg..., and the actin images his3-A1.jpg, his3-A2.jpg... and put them in the folder called his3. Cell wall images may be analyzed alone, but in this case no data related to the cell nucleus or actin are obtained.

## **2 Preparation to run the program – Installation of Java**

Since CalMorph is a Java program, install JRE (Java Runtime Environment) to run Java programs in advance. If not already installed on your computer, download and install the Java 2

Runtime Environment, version 1.4.2 or higher from <http://java.sun.com>.

### 3 Execution of CalMorph

CalMorph can be run on Linux, Mac OS X, etc. in addition to various versions of MS Windows provided that J2SE is installed. After downloading the file “CalMorph.jar” from <http://scmd.gi.k.u-tokyo.ac.jp/datamine/calmorph/>, you can launch CalMorph by simply double-clicking the icon. When CalMorph is started, input frames will be displayed.

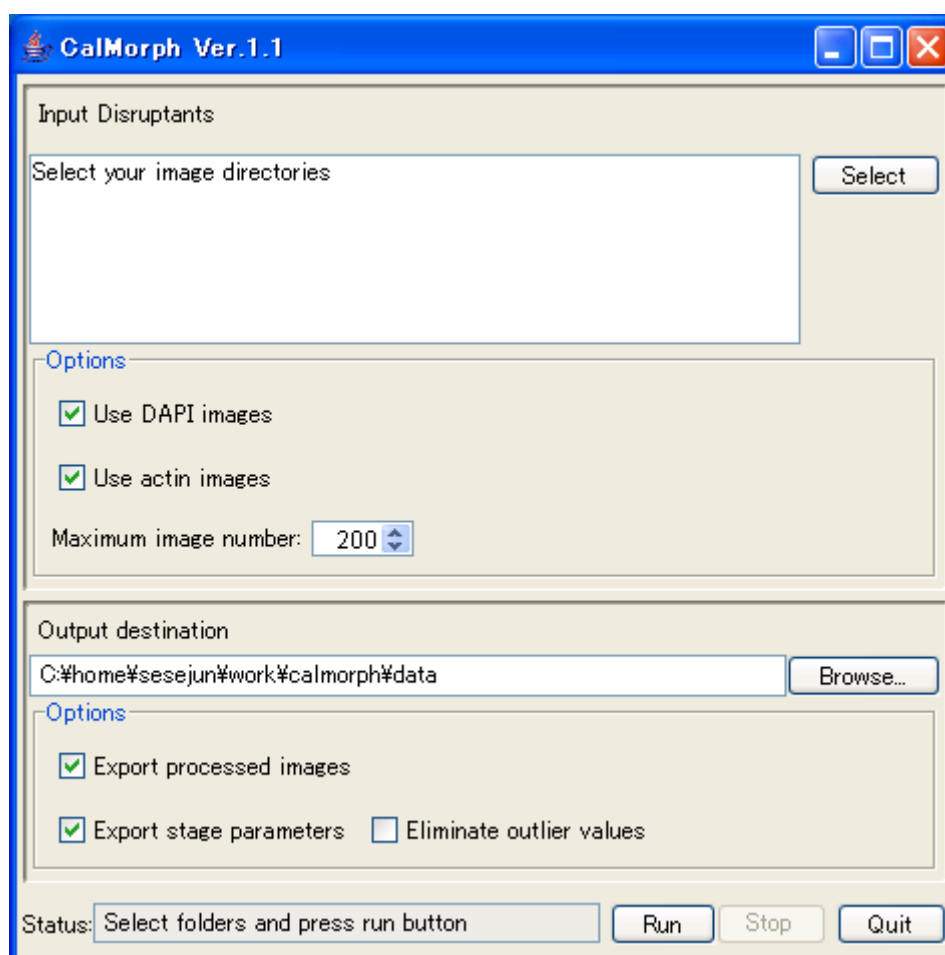


Figure 1. Input frames, for Windows XP. The appearance depends on the environment that the software is running in.

The folder name of the group to be analyzed is entered in the white input box located in the

upper frame. Click on the “select” button to the right of the input box to call the file browser and choose a folder. You can enter more than one group using control or command key. The check boxes under the input box are used to specify whether cell nucleus and actin images will be analyzed. Erase the check for “Use DAPI images” if you are not going to use cell nucleus images and the check for “Use actin images” if actin images are not used. “Maximum image number” in the frame is the upper bound of the image number to be analyzed. Increase this value if you are analyzing more than 200 images. It is usually not necessary to change the default value.

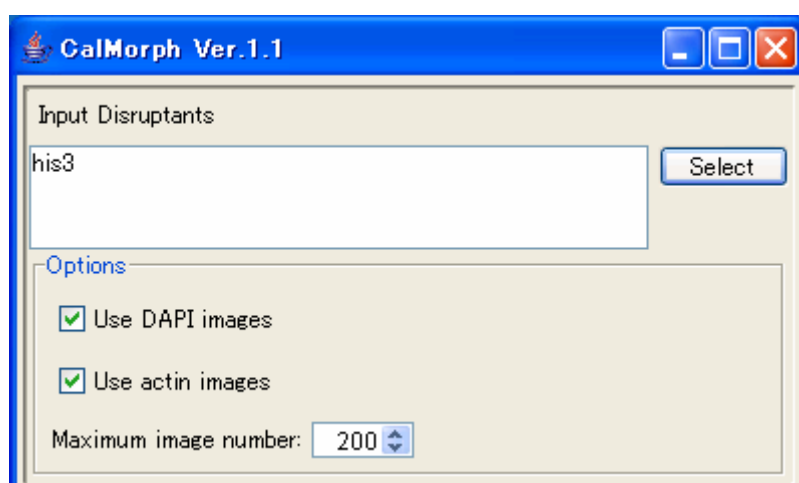


Figure 2. Upper frame, “his3” has been chosen as the group to be analyzed.

The folder name to which the results of the analysis will be output is entered in the box in the center frame (Figure 3). By default, this folder is called “data” and is located in the same folder as CalMorph. If this folder does not exist, it will be created. If you want to change the folder, click on the “Browse...” button to the right of the input box and choose a folder. When the analysis is performed, two new folders “cell” and “stage” will be created in the data folder .



“cell” folder contains folders with the name of the group to be analyzed is created in the selected folder and the result of the analysis is stored in that folder. You can specify whether to output an image of the results of the analysis by checking the “Export processed image” box under the input box. Remove this check if only numeric data are wanted and the resulting image is not required. “stage” folder will be created only when “Export stage parameters” box is checked. This folder contains biological parameter sheets classified by cell nucleus stages (See Section 5.4). If “Eliminate outlier values” box is checked, CalMorph will compute stage classified parameters without largest 1% and smallest 1% values because the values might be outlier caused from miss-identification of cell shapes.

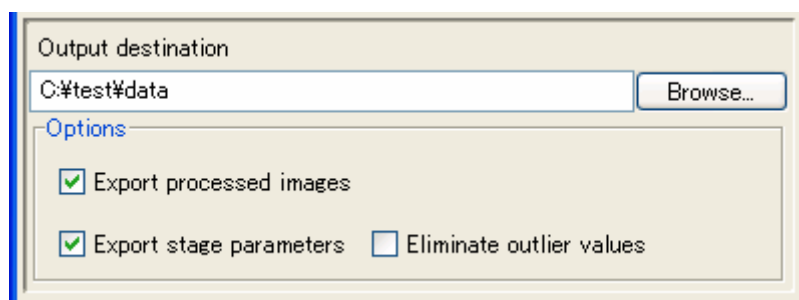


Figure 3. In the center frame, the output folder has been changed to the folder “test” located on drive C.

When you have finished making your choices for these settings, start the analysis by clicking on the ‘Run’ button in the bottom of the frame. The status of CalMorph is displayed in the “status” box on the left side of the button. Click on the ‘Reset’ button to stop the analysis before it is finished. Click on the ‘Quit’ button to exit CalMorph. If the analysis is stopped before it has been completed, the results up to the last image completed will be output.

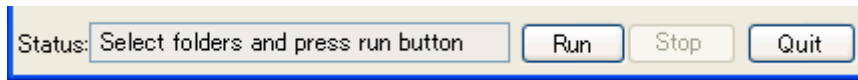


Figure 4. Lower frame, the analysis has just started, and the image being analyzed is displayed.

#### **4 Computation time required for the analysis**

The computation time required to analyze a set of images (cell wall, nucleus, and actin) is less than 10 seconds on a PC with a 1066-MHz Pentium III and 632 MB RAM. The image being analyzed is indicated in the status box in the bottom of the frame.

#### **5 Analysis results**

##### **5.1 Output file format**

When the analysis is complete, the data for individual cells is output to a folder, with the group name located in the folder specified in the output box. Five files are output with the following file names: (group name)\_conA\_basic.xls, (group name)\_dapi\_basic.xls, (group name)\_actin\_basic.xls, (group name)\_actin\_patch.xls, and (group name).xls. The files (group name)\_conA\_basic.xls, (group name)\_dapi\_basic.xls, and (group name)\_actin\_basic.xls contain the positions of the characteristic points of the cell wall, cell nucleus, and actin, respectively, in the images and basic parameters, such as the area. The file (group name)\_actin\_patch.xls contains the data on individual actin patches. These files can be opened using Microsoft Excel. Since the contents of these files are plain text with tab delimiters, ordinary text editors, such as WordPad, etc., can be used to examine their contents.

	A	B	C	D	E	F	G	H	I	J	K	L
1	image_num	cell_id	Cgroup	C1-1	C1-2	C2-1	C2-2	C3-1	C3-2	C4-1	C4-2	C5-1
2	1	0	complex	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
3	1	1	complex	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
4	1	2	no	[614,73]	[580,78]	[599,89]	[595,62]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
5	1	3	large	[56,79]	[93,80]	[74,95]	[75,64]	[57,87]	[68,94]	[62,90]	[47,115]	[43,96]
6	1	4	large	[475,99]	[439,96]	[459,82]	[456,113]	[476,99]	[470,86]	[473,92]	[497,81]	[490,99]
7	1	5	medium	[389,102]	[422,104]	[405,118]	[407,89]	[390,107]	[396,116]	[393,111]	[377,124]	[379,110]
8	1	6	large	[453,151]	[484,128]	[460,127]	[477,151]	[451,145]	[453,133]	[452,139]	[425,128]	[434,145]
9	1	7	no	[226,150]	[195,157]	[214,167]	[208,140]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
10	1	8	complex	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
11	1	9	small	[550,174]	[585,175]	[567,191]	[568,158]	[551,179]	[559,190]	[555,184]	[543,193]	[544,181]
12	1	10	complex	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
13	1	11	complex	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
14	1	12	no	[133,298]	[157,319]	[153,299]	[136,318]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
15	1	13	no	[209,309]	[213,337]	[223,322]	[198,325]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
16	1	14	complex	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
17	1	15	large	[330,363]	[337,327]	[320,342]	[348,348]	[325,362]	[320,350]	[322,356]	[301,367]	[316,370]
18	1	16	no	[39,336]	[14,360]	[37,359]	[16,338]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
19	1	17	large	[563,353]	[600,348]	[583,365]	[579,336]	[566,361]	[576,365]	[571,363]	[562,387]	[554,371]
20	1	18	small	[511,405]	[521,439]	[532,417]	[500,427]	[525,408]	[533,417]	[529,412]	[536,407]	[529,403]
21	1	19	no	[374,421]	[344,427]	[361,438]	[356,411]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]
22	1	20	medium	[661,459]	[681,430]	[659,436]	[683,453]	[656,456]	[656,444]	[656,450]	[634,450]	[645,460]
23	1	21	no	[188,454]	[177,480]	[194,472]	[172,462]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]	[-1,-1]

Figure 5. Sample output (his3\_conA\_basic.xls).

When the “Export processed image” check box is checked, images showing the analysis result are output to the (group name) folder.

If input images have not been processed properly for some reason, they may be excluded from the output. In such cases, a file exclusion\_log.txt containing the excluded image numbers and listing the possible causes will be created in the (group name) folder.

## 5.2 Description of each parameter

The results of the analysis are explained here. Here, “neck” is the part of a cell where a mother cell and bud separate. The units for length, area, etc. are numbers of pixels. The brightness of each point is expressed as an integer from 0 to 255, with larger numbers meaning higher brightness. The position of each characteristic point is expressed using the coordinate system with (0,0) at the upper-left corner of each image and (696,520) at the lower-right. The values -1 or (-1, -1) in the result indicate data that were not obtainable or that are non-existent.

### 5.3 Description of Basic Parameters

Basic Parameters are the ones extracted directly from the images, such as coordinate points critical for description of budding yeast cell morphology, area, brightness, etc. These parameters are calculated for each cell or each actin patch. Results are shown in file sets named “\_conA\_basic.xls”, “\_actin\_basic.xls”, “\_actin\_patch.xls”, and “\_dapi\_basic.xls”, according to the images they are extracted.

#### 5.3.1 Data items in (group name)\_conA\_basic.xls

- image\_number: The cell picture image number.
- cell\_id: The cell number assigned in the analysis.
- Cgroup: The cell cycle phase with respect to bud size. The phase of the cells without a bud is “no” and becomes “small”, “medium”, and “large” as the buds get larger. If the analysis failed because the cell shape was too complex to recognize, the cell cycle phase will be “complex”.

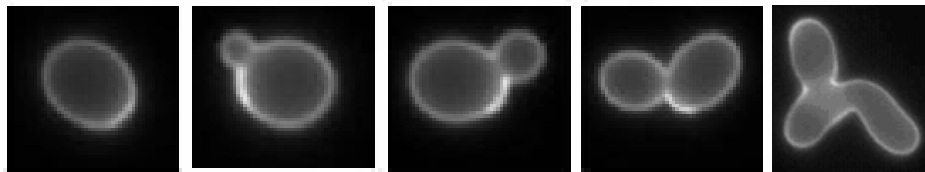


Figure 6. Examples of cells that belong to the five Cgroups: from left to right, no, small, medium, large, and complex.

- **C1-1, C1-2:** The ends of the major axis of the mother cell approximated by an ellipse. For a cell with a bud, the point closer to the midpoint of the neck is C1-1.
- **C2-1, C2-2:** The ends of the minor axis of the mother cell approximated by an ellipse. For a cell with a bud, the point closer to the midpoint of the neck is C2-1.

- C3-1, C3-2: The two ends of the neck of cells with a bud. The one closer to C1-1 is C3-1.
- C4-1, C4-2: The midpoint of the neck and the tip of a bud of cells with a bud, respectively.
- C5-1, C5-2: The ends of the minor axis of the ellipse with the major axis connecting C4-1 and C4-2 to approximate a bud of the cell. The one closer to C3-1 is C5-1.

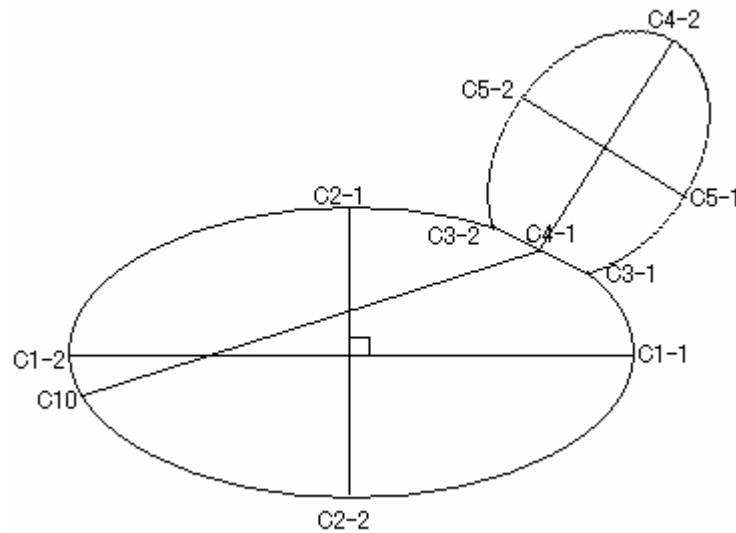


Figure 7. The meanings of C1..., C5, and C10.

- C6, C7: The points on the cell wall with the highest and lowest brightness, respectively.
- C8, C9: The thickest and thinnest points on a cell wall (not very accurate), respectively.
- C10: The point on the mother cell wall that is farthest from the mid-point of the neck of a cell with a bud.
- C11-1, C11-2: The areas of the mother cell and bud, respectively.
- C12-1, C12-2: The circumferences of the mother cell and bud, respectively.
- C13: The order of the elliptical approximation of the mother cell. (This is smaller when the actual shape is closer to an ellipse and larger when there is greater deviation from an elliptical shape.)

### 5.3.2 Data items in (group name)\_actin\_basic.xls

- image\_number, cell\_id and Cgroup: The same as in (group name)\_conA\_basic.xls.

- Agroup: Cell cycle phase with respect to the actin state. There are states a, b, c (api), d (iso), e, f, and n. They are output in (group name)\_actin\_basic.xls as A, B, api, iso, E, F, and N, respectively. Of these, a, b, c (api), d (iso), e, and f are normal states matching the natural cell cycle, while n is abnormal. The states are as follows:
  - a: Cells with no actin localization and no bud.
  - b: Cells with actin localization, but no bud.
  - c (api): Cells with actin localization around the tip of the bud (APIcal growth).
  - d (iso): Cells with actin spreading over the entire bud (ISOtropic growth).
  - e: Cells with actin spreading over the entire mother cell and bud.
  - f: Cells with actin localization around the neck.
  - n: Cells with no actin.

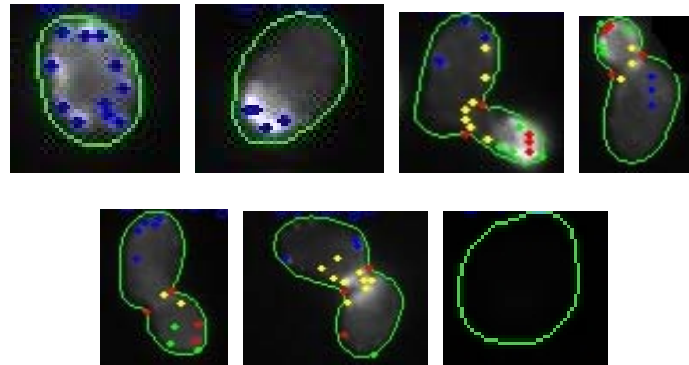


Figure 8. Examples of cells that belong to the seven Agroups (the cell outline is in green; dots are actin patches) in the order a, b, c (api), d (iso), e, f, and n from the upper-left.

- A2-1, A2-2, and A2-3: Center of gravity of the actin region (where actin is observed, *i.e.*, an area with a brightness greater or equal to a certain value) in a mother cell, bud, and whole cell, respectively.
- A3-1, A3-2, and A3-3: Center of gravity of the actin patches in a mother cell, bud, and whole cell, respectively.

- A4-1, A4-2, and A4-3: The center of gravity weighted by the biquadrate of the brightness of the actin region in a mother cell, bud, and whole cell, respectively.
- A5-1, A5-2, and A5-3: The center of gravity weighted by the biquadrate of the brightness of the actin patches in a mother cell, bud, and whole cell, respectively.
- A6-1, A6-2, and A6-3: The farthest point from the midpoint of the neck in the actin region in a mother cell, bud, and whole cell, respectively.
- A7-1 and A7-2: The area of the actin region in a mother cell and bud, respectively.
- A8-1 and A8-2: The sum of the actin brightness in a mother cell and bud, respectively.
- A9: The ratio of the actin region on a line segment connecting the necks.

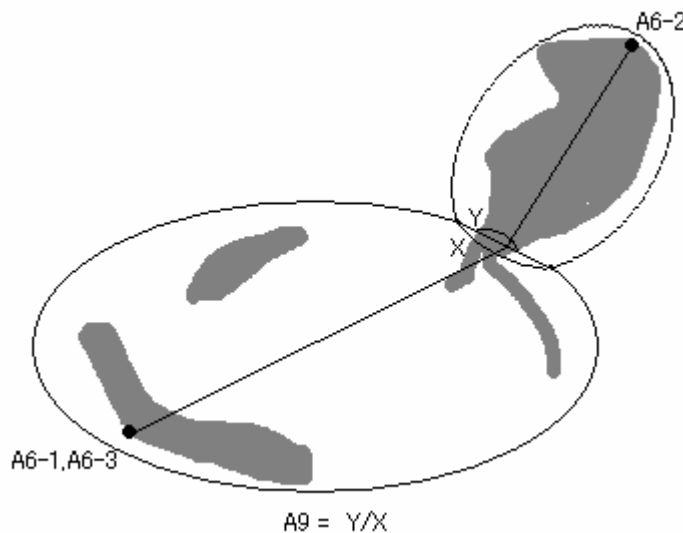


Figure 9. Meanings of A6 and A9 (the gray areas are actin regions).

### 5.3.3 Data items in (group name)\_actin\_patch.xls

- image\_number and cell\_id: The same as for (group name)\_conA\_basic.xls.
- position: Center of the actin patch (where the brightness is a local maximum).
- size: Area of the actin patch.
- brightness: Brightness at the center of the actin patch.

### 5.3.4 Data items in (group name)\_dapi\_basic.xls

- image\_number, cell\_id and Cgroup: The same as for (group name)\_conA\_basic.xls.
- Dgroup: Cell cycle phase with respect to the nucleus condition. There are states A, A1, B, C, D, E, and F. Of these, A, A1, B, and C are normal states matching the natural cell cycle, while D, E, and F are abnormal. The states are as follows:

A: Cells with one nucleus without a bud.

A1: Cells with one nucleus in the mother cell with a bud.

B: Cells in which the nucleus is dividing at the neck.

C: Cells with one nucleus each in the mother cell and bud.

D: Cells with two nuclei and no bud or those with two nuclei together in either the mother cell or bud.

E: Cells with no nucleus or with more than two nuclei.

F: Cells with one nucleus only in the bud.

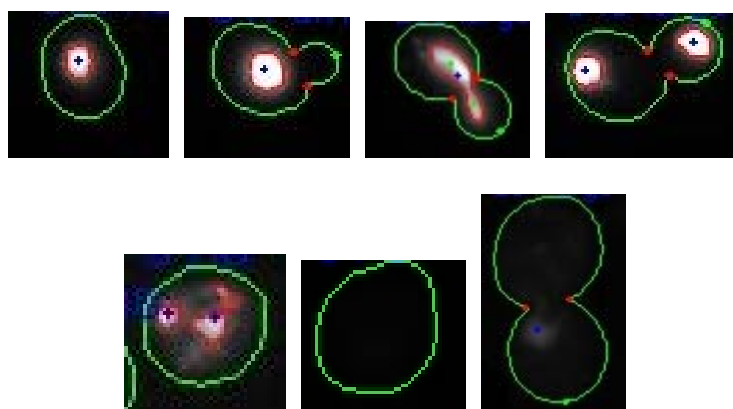


Figure 10. Examples of cells belonging to the seven Dgroups (the cell outline is in green, the nucleus periphery in red, and the nucleus center in blue) in the order A, A1, B, C, D, E, and F from the upper-left.



Hereafter, the nucleus in the mother cell means those nuclei in the mother cell in Dgroups A, A1, C, and the part of the nucleus in the mother cell of B cells. The nucleus in the bud means the nucleus in the bud in C cells and the part of the nucleus in the bud in B cells. The whole nucleus means the whole nucleus in A, A1, and B cells.

- D1-1, D1-2, and D1-3: Nucleus center of gravity in the mother cell, bud, and whole cell, respectively. If gravity center is in the mother cell and bud in B cells, it is output as D1-3-1 and D1-3-2, respectively.
- D2-1, D2-2, and D2-3: The brightest point in the nucleus in the mother cell, bud, and whole cell, respectively. If the brightest point is in the mother cell and bud in B cells, it is output as D2-3-1 and D2-3-2, respectively.
- D3-1, D3-2, and D3-3: The point on the nucleus outline farthest from the nucleus center of gravity in the mother cell, bud, and whole cell, respectively.
- D4-1, D4-2, and D4-3: The point on the nucleus outline farthest from D3 in the mother cell, bud, and whole cell, respectively.
- D5-1, D5-2, and D5-3: The point on the nucleus outline farthest from the nucleus center of gravity, on the line through the nucleus center of gravity perpendicular to line D3D4 in the mother cell, bud, and whole cell, respectively.

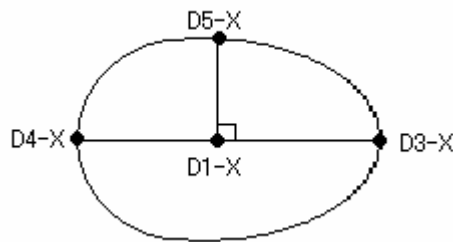


Figure 11. Meanings of D3-X, D4-X, and D5-X. The figure shows the outline of the nucleus, where D1-X is the center of gravity.

- D6-1 and D6-2: Intersection between the line connecting the nucleus center of gravity and the midpoint of the neck, and the outline of the nucleus of the mother cell and bud, respectively.

- D7: Intersection between the line connecting the nucleus center of gravity of the mother cell and the point on the mother cell wall farthest (C10) from the midpoint of the neck, and the outline of the nucleus in the mother cell.
- D8: Intersection between the line connecting the nucleus center of gravity of the bud and the tip of the bud, and the outline of the nucleus in the bud.

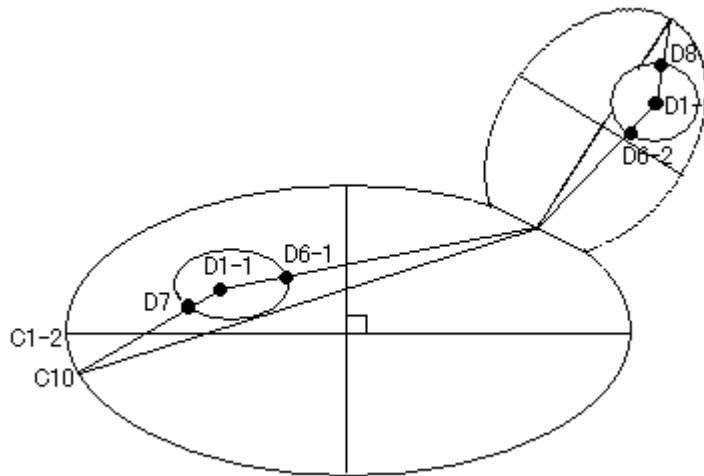


Figure 12. Locations of D6, D7, and D8

- D9-1 and D9-2: The respective points on the outline of the mother cell (or bud) intersected by the line connecting the center of the mother cell (or bud) and the nucleus center of gravity of the mother cell (or bud).
- D10-1 and D10-2: The respective points on the outline of the mother cell (or bud) intersected by the line connecting the center of the mother cell (or bud) and the brightest point in the nucleus of the mother cell (or bud).

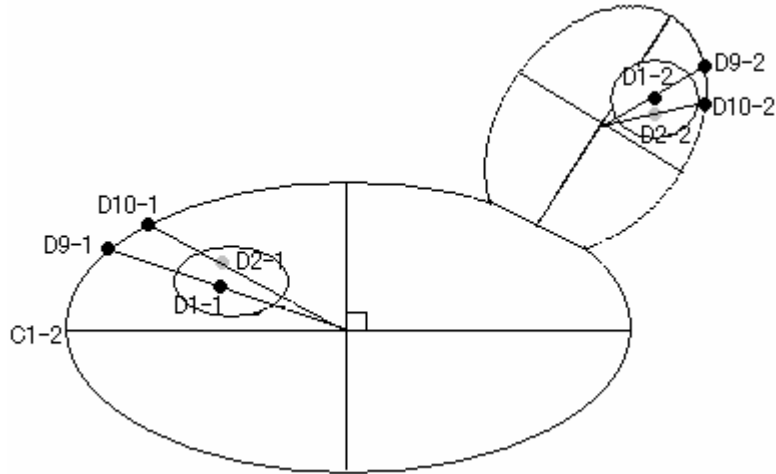


Figure 13. Locations of D9 and D10

- D11-1 and D11-2: The respective points on the outlines of the mother cell (or bud) intersected by the line connecting the nucleus center of gravity of the mother cell (or bud) and the midpoint of the neck.
- D12-1 and D12-2: The respective points on the outlines of the mother cell (or bud) intersected by the line connecting the brightest point in the nucleus of the mother cell (or bud) and the midpoint of the neck.
- D13-1 and D13-2: For the cells in Dgroup B, the respective points on the outlines of the mother cell (or bud) intersected by the line connecting either D3-3 or D4-3, whichever is inside the mother cell (or D3-3 or D4-3, whichever is inside the bud), and the midpoint of the neck.

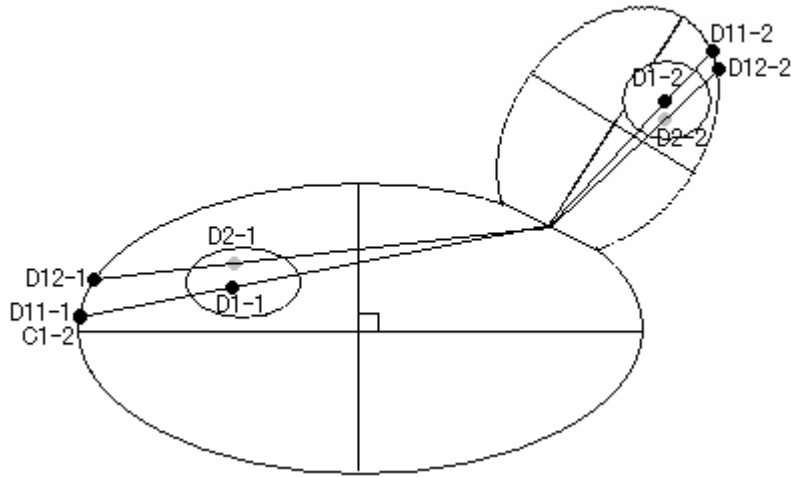


Figure 14. The locations of D11 and D12 (cells in Dgroup C)

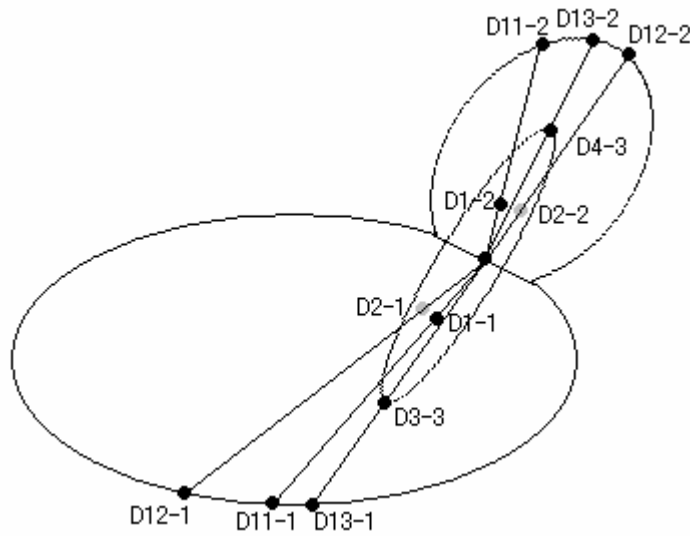


Figure 15. The locations of D11, D12, and D13 (cells in Dgroup B)

- D14-1, D14-2, and D14-3: Areas of the nucleus in the mother cell, bud, and whole cell, respectively.
- D15-1, D15-2, and D15-3: Sum of the nucleus brightness of the mother cell, bud, and whole cell, respectively.
- D16-1, D16-2, and D16-3: The highest brightness in the nucleus of the mother cell, bud, and whole cell, respectively.
- D17-1, D17-2, and D17-3: Elliptical approximation of the nuclei. (The value is smaller if the

actual shape is closer to a true ellipse and becomes larger as it deviates from being elliptical.)

## **5.4 Description of Biological Parameters**

### **5.4.1 Overview of Biological Parameters**

As described in the former chapters, we set various points those are useful for description of budding yeast cell morphology as Basic Parameters. Calculating from the coordinate points of them, numerous parameters can be set. Among those parameters out of number, we choose 501 parameters as “Biological Parameters” that seem to be significant to describe yeast cell morphology. Biological Parameters can be divided into categories based on several criteria.

Criteria 1. Parameters extracted from ConA-images (shown as capital letter “C”), those from actin-images (“A”), and those from DAPI-images (“D”). In total, 133 C parameters, 96 A parameters, 272 D parameter are set. The first letter of ID indicates from which image that parameter was extracted.

Criteria 2. Parameters calculated from each cell, and those from whole population as ratio. The former parameters are divided into “stages” as described in Criteria 3. Further, coefficients of variation (CV) for those parameters are also calculated. The latter parameters are expressed as “Total\_stage” parameters. In total, parameters for each cell are 241, CV parameters are 220, and Total\_stage parameters are 40. CV parameters are expressed as “NCV###” (N is A, C or D). Total stage parameters are C119-125, A105-119, and D199-D216.

Criteria 3. Parameters common to several “stages”, and those only used for the cells at special “stages”. This classification is introduced in order to exclude ambiguity caused by mingling the cells with disparate morphology. This issue is further discussed below.

In the wild type cells of budding yeast, cell wall, nucleus, and actin cooperatively change their morphology, location, and localization, respectively, as cell cycle progresses.

Although CalMorph has a virtue of extraction of each cell information in unsynchronized culture, and the mean value of parameters may indicate the morphological feature of a specimen, it seems to have less meaning if, for example, the mean value of cell size is calculated from the mixed population including cells with bud and without bud. So, in order to concern with the morphological changes accompanied with cell cycle progression, CalMorph is prepared to divide each cell automatically into the “stages”, and the mean value is calculated from the cells at the same stages. Note that the standard was set originally based on morphological changes seen in wild type cells.

Stage-division process is based on two types of standards. First, it is determined whether the cell has a bud or no bud. Then, nuclear number and location is concerned. Based on these standards, cells are categorized as:

Stage A: The cell with no bud and one nucleus.

Stage A1: The cell with a bud and one nucleus in the mother cell.

Stage B: The cell with a bud and one nucleus at the neck.

Stage C: The cell with a bud and two nuclei, one in the mother and one in the daughter.

In wild type, Stage A cells are between G1 phase and S phase, Stage A1 cells, between S and G2, Stage B cells, at nuclear division phase, and Stage C cells, at cytokinesis. Asynchronous cultures, however, contain not so many Stage B cells that it is difficult to obtain statistically meaningful data for Stage B alone. Therefore, after divided into stages, Stage A1 cells and Stage B cells are to be concerned as one novel category, Stage A1B cells. Finally, the number of Stage A parameters are 73; Stage A1B, 153; and Stage C, 235.

#### **5.4.2 Description of each Biological Parameter**

Here we describe the meaning and definition of Biological Parameters, besides CV parameters and those described as Basic Parameters in the chapter 5.3.1-5.3.3 (C11-13, A7-9, and D

14-17). To make description simple, novel points are set besides Basic Parameters.

C1: The center of the mother cell. The point is determined as crossing point of the line between C1-1 and C1-2, and that between C2-1 and C2-2.

C2: The center of bud. The point is determined as crossing point of the line between C4-1 and C4-2, and that between C5-1 and C5-2.

The units of Biological Parameters for length, distance are “pixel”, those for area are “pixel<sup>2</sup>”.

The ratio and brightness parameters have no unit.

#### 5.4.2.1 Biological parameters calculated from Basic parameters of ConA-Images

Note that the parameters concerning bud are not calculated for Stage A cells.

C11-13: See chapter 5.3.1.

**C101:** Whole cell size. The sum of mother and daughter cell size (C11-1+C11-2).

C102: Whole cell outline length. The sum of mother and daughter cell outline length (C12-1+C12-2).

**C103:** Long axis length in mother cell. The length between C1-1 and C1-2.

**C104:** Short axis length in mother cell. The length between C2-1 and C2-2.

C105: Neck position. The angle between the line joining C1-1 and C1, and that joining C1 and C4-1.

C106: Bud direction: The angle between the extension of the line joining C4-1 and C4-2, and mother long axis (the line between C1-1 and C1-2).

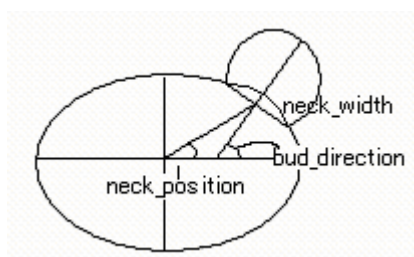


Figure 16. Meanings of bud direction, neck position, and neck width.

C107: Long axis length in bud: The length between C4-1 and C4-2.

C108: Short axis length in bud: The length between C5-1 and C5-2.

C109: Neck width: The length between C3-1 and C3-2.

C110: Distance between bud tip and mother long axis extension. The length of the line, which is perpendicular to the mother long axis (the line between C1-1 and C1-2) dropped from C4-2.

C111: Distance between bud tip and mother short axis extension. The length of the line, which is perpendicular to the mother short axis (the line between C2-1 and C2-2) dropped from C4-2.

C112: Distance between middle point of neck and mother center. The length of the line between C1 and C4-1.

C113: Distance between bud tip and mother long axis through middle point of neck. The length of the line joining C4-2 and the point where extension of the line between C4-1 and C4-2 crosses mother long axis.

C114: Bud axis ratio. The ratio of bud long axis (C107) to bud short axis (C108).

C115: Mother axis ratio. The ratio of mother long axis (C103) to mother short axis (C104).

C116: Axis ratio ratio. The ratio of bud axis ratio (C114) to mother axis ratio (C115).

C117: Cell outline ratio. The ratio of mother cell outline length (C12-1) to bud cell outline length (C12-2).

C118: Cell size ratio. The ratio of bud cell size (C11-2) to mother cell size (C11-1).

C119: No bud ratio. The ratio of no bud cells within the cells in the specimen.

C120: Small bud ratio. The ratio of small bud cells within the cells in the specimen.

C121: Medium bud ratio. The ratio of medium bud cells within the cells in the specimen.

C122: Large bud ratio. The ratio of large bud cells within the cells in the specimen.



C123: Small bud ratio to budded cells. The ratio of small bud cells within budded cells.

C124: Medium bud ratio to budded cells. The ratio of medium bud cells within budded cells.

C125: Large bud ratio to budded cells. The ratio of large bud cells within budded cells.

C126: Brightness difference of cell wall. The difference between the brightness at the brightest point and that at the darkest point on the outline of cell wall (C6 minus C7).

C127: Thickness difference of cell wall. The difference in the thickness of cell wall outline between that at the thickest point and that at the thinnest point (C8 minus C9).

C128: Distance between middle point of neck and mother hip. The distance between C4-1 and C10.

#### **5.4.2.2 Biological parameters calculated from Basic parameters of Actin-Images**

Note that the parameters for Actin A, Actin B, and no bud cells are calculated only for Stage A cells, and those for other Actin categories and budded cells are for Stage A1B, C cells.

A7-A9: See chapter 5.3.2.

A101: Actin region ratio in whole cell. The ratio of actin region area (A7-1+A7-2) to whole cell area (C11-1+C11-2).

A102: Bud actin region ratio to total region. The ratio of actin region area in bud (A7-2) to that in whole actin region (A7-1+A7-2).

A103: Relative gravity center of weighted actin region in mother. A perpendicular line is dropped from A4-1 on the line between C4-1 and C10. A103 is the distance between the intersection point and C4-1 relative to the distance between C4-1 and C10.

A104: Relative gravity center of weighted actin region in bud. A perpendicular line is dropped from A4-2 on the line between C4-1 and C4-2. A104 is the distance between the intersection point and C4-1 relative to the distance between C4-1 and C4-2.

A105: Actin a ratio. The ratio of the cells with actin a state (cells with no actin localization and

no bud) in the specimen.

A106: Actin b ratio. The ratio of the cells with actin b state (cells with actin localization, bud no bud) in the specimen.

A107: Actin c (api) ratio. The ratio of the cells with actin c (api) state (cells with actin localization around the tip of the bud) in the specimen.

A108: Actin d (iso) ratio. The ratio of the cells with actin d (iso) state (cells with actin spreading over the entire bud) in the specimen.

A109: Actin e ratio. The ratio of the cells with actin e state (cells with actin spreading over the entire mother cell and bud) in the specimen.

A110: Actin f ratio. The ratio of the cells with actin f state (cells with actin localization around the neck.) in the specimen.

A111: Actin ae ratio. The ratio of the cells with no actin localization (sum of A105 and A109) in the specimen.

A112: Actin bcd ratio. The ratio of the cells with actin localization except for around the neck (sum of A106, A107, and A108) in the specimen.

A113: Actin n ratio. The ratio of the cells with actin n state (cells with no actin) in the specimen.

A114: Actin a ratio to no bud cells. The ratio of the cells with actin a state within no bud cells.

A115: Actin b ratio to no bud cells. The ratio of the cells with actin b state within no bud cells.

A116: Actin c (api) ratio to budded cells. The ratio of the cells with actin c (api) state within budded cells.

A117: Actin d (iso) ratio to budded cells. The ratio of the cells with actin d (iso) state within budded cells.

A118: Actin e ratio to budded cells. The ratio of the cells with actin e state within budded cells.

A119: Actin f ratio to budded cells. The ratio of the cells with actin f state within budded cells.

A120: Total length of actin patch link. It is possible to connect actin patches by single

continuous line. A120 is the minimal length of that line.

A121: Maximal distance between patches. The distance between the most distant patches.

A122: Number of bright actin patches. The number of actin bright points those are recognized as “patch”.

A123: Ratio of actin patches to actin region. The ratio of the sum area of actin patches to whole actin region.

#### **5.4.2.3 Biological parameters calculated from Basic parameters of DAPI-Images**

Note that definitions of some nuclear Basic Parameters are set specifically for the cells at each stage. Biological Parameters using such Basic Parameters have separate ID, although they have the same description in words.

D14-D17: See chapter 5.3.3.

D102 (Stage A only): Distance between nuclear gravity center and mother tip. The distance between D1-1 and the nearer cell tip (C1-1 or C1-2).

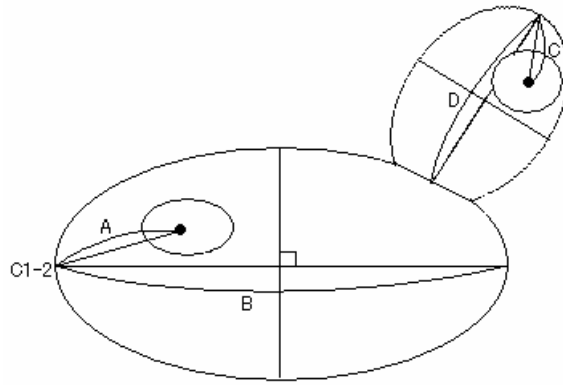
D103 (Stage C only): Distance between nuclear gravity center in mother and mother tip. The distance between D1-1 and C1-2.

D104 (Stage A1B only): Distance between nuclear gravity center and mother tip. The distance between D1-1 and C1-2.

D105 (Stage A only): Ratio of D102 to C103.

D106 (Stage C only): Ratio of D103 to C103.

D107 (Stage A1B only): Ratio of D104 to C103.



D105, 106, 107: A/B  
D123: C/D

Figure 17. The meanings of D105, D106, D107 and D123.

D108 (Stage C only): Distance between nuclear gravity center in mother (D1-1) and middle point of neck (C4-1).

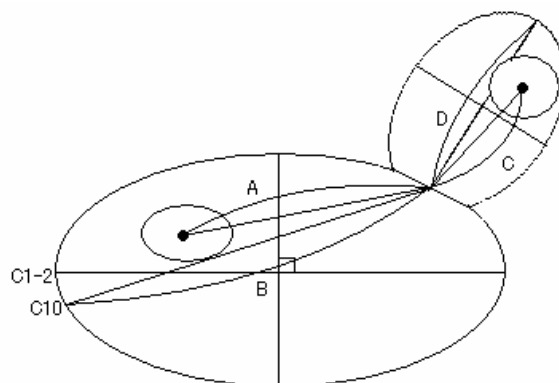
D109 (Stage C only): Distance between nuclear gravity center in bud (D1-2) and middle point of neck (C4-1).

D110 (Stage A1B only): Distance between nuclear gravity center (D1-1) and middle point of neck (C4-1).

D112 (Stage C only): Ratio of D108 to C128.

D113 (Stage C only): Ratio of D109 to C107.

D114 (Stage A1B only): Ratio of D110 to C128.



D112, D114: A/B  
D113: C/D

Figure 18. The meanings of D112, D114 and D113.

D116 (Stage C only): Distance between two nuclear gravity centers through middle point of neck. The sum of distances between D1-1 and C4-1 and between D1-2 and C4-1.

D117 (Stage A and C only): Distance between nuclear gravity center in mother (D1-1) and mother center (C1).

D118 (Stage A1B only): Distance between nuclear gravity center (D1-1) and mother center (C1).

D119 (Stage C only): Distance between nuclear gravity center in bud (D1-2) and bud center (C2).

D121 (Stage C only): Distance between nuclear gravity center in bud (D1-2) and bud tip (C4-2).

D123 (Stage C only): Ratio of D121 to C107.

D125 (Stage C only): Distance between nuclear gravity center in mother (D1-1) and mother hip (C10).

D126 (Stage A1B only): Distance between nuclear gravity center (D1-1) and mother hip (C10).

D127 (Stage A only): Distance between nuclear brightest point (D2-1) and the nearer cell tip (C1-1 or C1-2).

D128: (Stage C only): Distance between nuclear brightest point in mother (D2-1) and mother tip (C1-2).

D129 (Stage A1B only): Distance between nuclear brightest point (D2-1) and mother tip (C1-2).

D130 (Stage C only): Distance between nuclear brightest point in mother (D2-1) and middle point of neck (C4-1).

D131 (Stage C only): Distance between nuclear brightest point in bud (D2-2) and middle point of neck (C4-1).

D132 (Stage A1B only): Distance between nuclear brightest point (D2-1) and middle point of

neck (C4-1).

D134 (Stage C only): Distance between two nuclear brightest points through middle point of neck. (Sum of distances between D2-1 and C4-1 and between D2-2 and C4-1).

D135 (Stage A and C only): Distance between nuclear brightest point in mother (D2-1) and mother center (C1).

D136 (Stage A1B only): Distance between nuclear brightest point (D2-1) and mother center (C1).

D137 (Stage C only): Distance between nuclear brightest point in bud (D2-2) and bud center (C2).

D139 (Stage C only): Distance between nuclear brightest point in bud (D2-2) and bud tip (C4-2).

D141 (Stage C only): Distance between nuclear brightest point in mother (D2-1) and mother hip (C10).

D142 (Stage A1B only): Distance between nuclear brightest point (D2-1) and mother hip (C10).

D143 (Stage A1B and C only): Distance between nuclear outline point D6-1 and middle point of neck (C4-1).

D144 (Stage C only): Distance between nuclear outline point D6-2 in bud and middle point of neck (C4-1).

D145 (Stage A1B and C only): Distance between nuclear outline point D7 in mother and mother hip (C10).

D146 (Stage C only): Distance between nuclear outline point D8 in bud and bud tip (C4-2).

D147: Relative distance of nuclear gravity center in mother (D1-1) to mother center (C1). The ratio of the distance between D1-1 and C1 to that between D9-1 and C1.

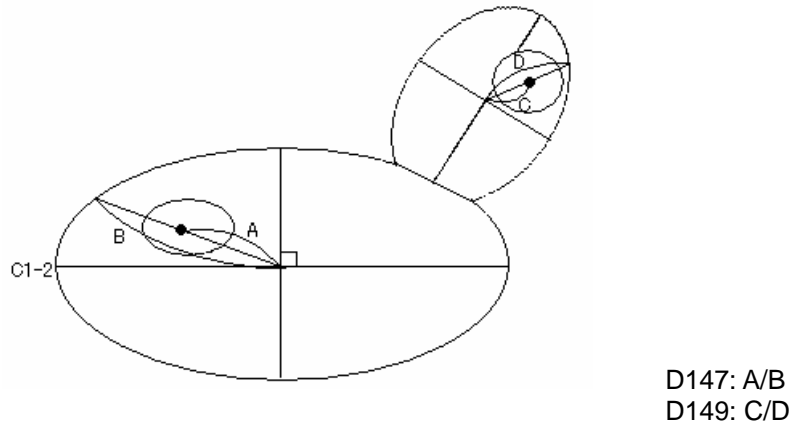


Figure 19. The meanings of D147 and D149.

D148: Relative distance of nuclear brightest point in mother (D2-1) to mother center (C1). The ratio of D2-1 and C1 to D10-1 and C1.

D149 (Stage C only): Relative distance of nuclear gravity center in bud to bud center. The ratio of the distance between D1-2 and C2 to that between D9-2 and C2.

D150 (Stage C only): Relative distance of nuclear brightest point in bud to bud center. The ratio of the distance between D2-2 and C2 to that between D10-2 and C2.

D151 (Stage C only): Ratio of distance between each nucleus and middle point of neck. The ratio of the distance between D6-2 and C4-1 to that between D6-1 and C4-1.

D152 (Stage A1B and C only): Mobility of nucleus in mother. The ratio of the distance between D6-1 and C4-1 to that between D7 and C10.

D153 (Stage C only): Mobility of nucleus in bud. The ratio of the distance between D6-2 and C4-1 to that between D8 and C4-2.

D154: Angle between C1D1-1 and C1C1-2.

D155: Angle between C1D2-1 and C1C1-2.

D156 (Stage C only): Angle between C2D1-2 and C2C4-2.

D157 (Stage C only): Angle between C2D2-2 and C2C4-2.

D158 (Stage C only): Angle between D1-1D1-2 and C1-1C1-2.

D159 (Stage C only): Angle between D2-1D2-2 and C1-1C1-2.

D161 (Stage A1B only): Angle between D3-1D4-1 and C1-1C1-2 or between D3-3D4-3 and C1-1C1-2.

D162 (Stage C only): Angle between D1-1D1-2 and C1C4-1.

D163 (Stage C only): Angle between D2-1D2-2 and C1C4-1.

D165 (Stage A1B only): Angle between D3-1D4-1 and C1C4-1 or between D3-3D4-3 and C1C4-1.

D166 (Stage C only): Angle between D1-1D1-2 and C4-1C4-2.

D167 (Stage C only): Angle between D2-1D2-2 and C4-1C4-2.

D169 (Stage A1B and C only): Angle between C4-1D1-1 and C4-1C1.

D170 (Stage A1B and C only): Angle between C4-1D2-1 and C4-1C1.

D172 (Stage A1B only): Angle between C4-1D4 and C4-1C1.

D173 (Stage A and C only): Maximal distance between nuclear gravity center (D1-1) and nuclear outline in mother (D3-1).

D174 (Stage C only): Maximal distance between nuclear gravity center (D1-2) and nuclear outline in bud (D3-2).

D175 (Stage A1B only): Maximal distance between nuclear gravity center (D1-1 or D1-2) and nuclear outline (D3-3).

D176 (Stage A and C only): Nuclear long axis length in mother. The length of the line between D3-1 and D4-1.

D177 (Stage C only): Nuclear long axis length in bud. The length of the line between D3-2 and D4-2.

D178 (Stage A1B only): Nuclear long axis length. The length of the line between D3-3 and D4-3.

D179 (Stage A and C only): Nuclear minimum radius in mother. The length of the line between



D1-1 and D5-1.

D180 (Stage C only): Nuclear minimum radius in bud. The length of the line between D1-2 and D5-2.

D181 (Stage A1B only): Nuclear minimum radius. The length of the line between D1-3 and D5-3.

D182 (Stage A and C only): Nuclear axis ratio in mother. The ratio of D176 to D179..

D183 (Stage C only): Nuclear axis ratio in bud. The ratio of D177 to D180.

D184 (Stage A1B only): Nuclear axis ratio. The ratio of D178 to D181.

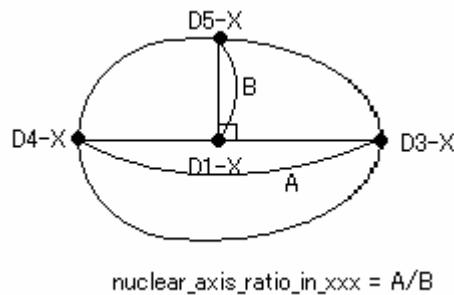


Figure 20. Meaning of nuclear axis ratio in mother/bud/cell. The figure shows the outline of a nucleus.

D185 (Stage C only) Total length of two straight segments D11-1C4-1 and D11-2C4-1.

D186 (Stage C only) Total length of two straight segments D12-1C4-1 and D12-2C4-1.

D188 (Stage A and C only): Distance between nuclear gravity center (D1-1) and brightest point in mother (D2-1).

D189 (Stage C only): Distance between nuclear gravity center (D1-2) and brightest point in bud (D2-2).

D190 (Stage A1B only): Distance between nuclear gravity center (D1-1 or D1-2) and brightest point (D2-1 or D2-2). The combination with minimal distance is indicated.

D191 (Stage A and C only): Average of nuclear brightness in mother. It is calculated by dividing sum of the brightness of the nucleus in mother (D15-1) by area of the nucleus in mother (D14-1).

D192 (Stage C only): Average of nuclear brightness in bud. It is calculated by dividing sum of the brightness of the nucleus in bud (D15-2) by area of the nucleus in bud (D14-2).

D193 (Stage A1B and C only): Average of nuclear brightness in whole cell. It is calculated by dividing sum of the brightness of the nucleus in whole cell (D15-3) by area of the nucleus in whole cell (D14-3).

D194 (Stage A and C only): Maximal intensity of nuclear brightness divided by average in mother. It is calculated by dividing the highest brightness in the nucleus in mother (D16-1) by D191.

D195 (Stage C only): Maximal intensity of nuclear brightness divided by average in bud. It is calculated by dividing the highest brightness in the nucleus in bud (D16-2) by D192.

D196 (Stage A1B and C only): Maximal intensity of nuclear brightness divided by average in whole cell. It is calculated by dividing the highest brightness in the nucleus in whole cell (D16-3) by D193.

**D197** (Stage C only): Ratio of nuclear size. The ratio of nuclear area in bud (D14-2) to nuclear area in mother (D14-1).

D198 (Stage C only): Ratio of nuclear brightness. The ratio of nuclear brightness in bud (D15-2) to nuclear brightness in mother (D15-1).

D199: Nuclear A ratio. The ratio of the cells with nuclear A state (cells with one nucleus without a bud) in the specimen.

D200: Nuclear A1 ratio. The ratio of the cells with nuclear A1 state (cells with one nucleus in the mother cell with a bud) in the specimen.

D201: Nuclear B ratio. The ratio of the cells with nuclear B state (cells in which the nucleus is

- dividing at the neck) in the specimen.
- D202: Nuclear C ratio. The ratio of the cells with nuclear C state (cells with one nucleus each in the mother cell and bud) in the specimen.
- D203: Nuclear D ratio. The ratio of the cells with nuclear D state (cells with two nuclei and no bud or those with two nuclei together in either the mother cell or bud) in the specimen.
- D204: Nuclear E ratio. The ratio of the cells with nuclear E state (cells with no nucleus or with more than two nuclei) in the specimen.
- D205: Nuclear F ratio. The ratio of the cells with nuclear F state (cells with one nucleus only in the bud) in the specimen.
- D206: Nuclear A ratio to no bud cells. The ratio of the cells with nuclear A state within no bud cells.
- D207: Nuclear A1 ratio to budded cells. The ratio of the cells with nuclear A1 state within budded cells.
- D208: Nuclear B ratio to budded cells. The ratio of the cells with nuclear B state within budded cells.
- D209: Nuclear C ratio to budded cells. The ratio of the cells with nuclear C state within budded cells.
- D210: Nuclear A ratio to nuclear AA1BC cells. The ratio of the cells with nuclear A state within nuclear A, A1, B, and C cells, excluding the cells with uncommon nuclear state.
- D211: Nuclear A1 ratio to nuclear AA1BC cells. The ratio of the cells with nuclear A1 state within nuclear A, A1, B, and C cells, excluding the cells with uncommon nuclear state.
- D212: Nuclear B ratio to nuclear AA1BC cells. The ratio of the cells with nuclear B state within nuclear A, A1, B, and C cells, excluding the cells with uncommon nuclear state.
- D213: Nuclear C ratio to nuclear AA1BC cells. The ratio of the cells with nuclear C state within nuclear A, A1, B, and C cells, excluding the cells with uncommon nuclear state.

D214: Nuclear A1 ratio to nuclear A1BC cells. The ratio of the cells with nuclear A1 state within A1, B, and C cells, excluding no bud cells and the cells with uncommon nuclear state.

D215: Nuclear B ratio to nuclear A1BC cells. The ratio of the cells with nuclear B state within A1, B, and C cells, excluding no bud cells and the cells with uncommon nuclear state.

D216: Nuclear C ratio to nuclear A1BC cells. The ratio of the cells with nuclear C state within A1, B, and C cells, excluding no bud cells and the cells with uncommon nuclear state.

### 5.5 Image showing the results of the cell wall analysis ((group name)-conA(image no.).jpg)

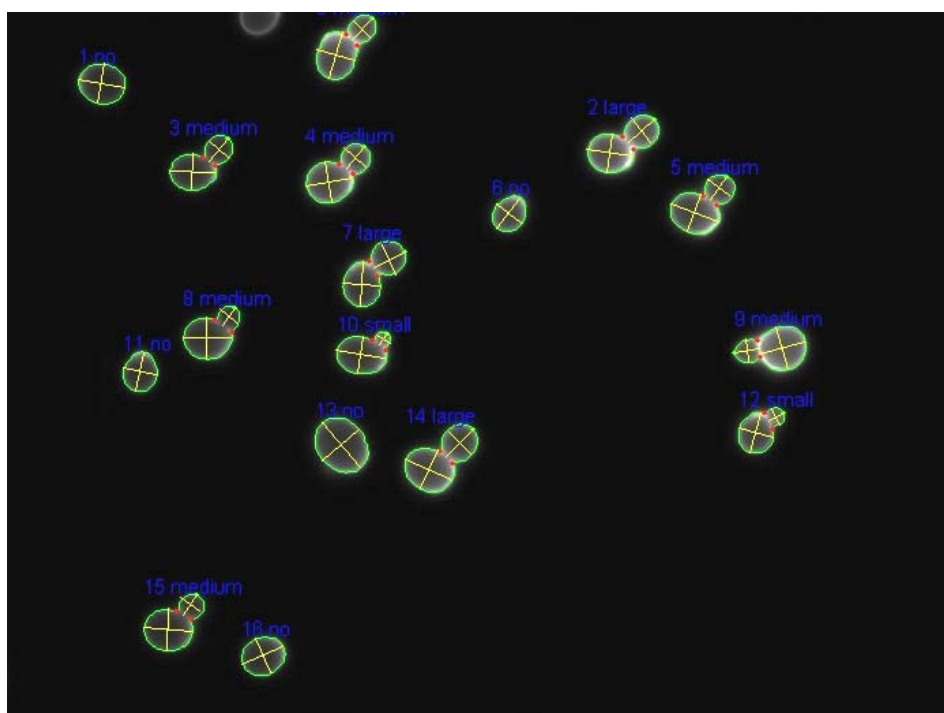


Figure 21. Example of a resulting image (Cell Wall).

- Blue letters: Cell numbers assigned in the analysis and cell cycle phases with respect to bud size (Cgroup).
- Green lines: Recognized cell walls.
- Yellow lines: Major and minor axes of the ellipses approximating the mother cells and buds.

- Green points (Cells with buds only): Tip of the bud.
- Red points (Cells with buds only): Boundary points between the mother cell and bud (neck).

## 5.6 Image showing the results of the nucleus analysis ((group name)-dapi(image no.)).jpg)

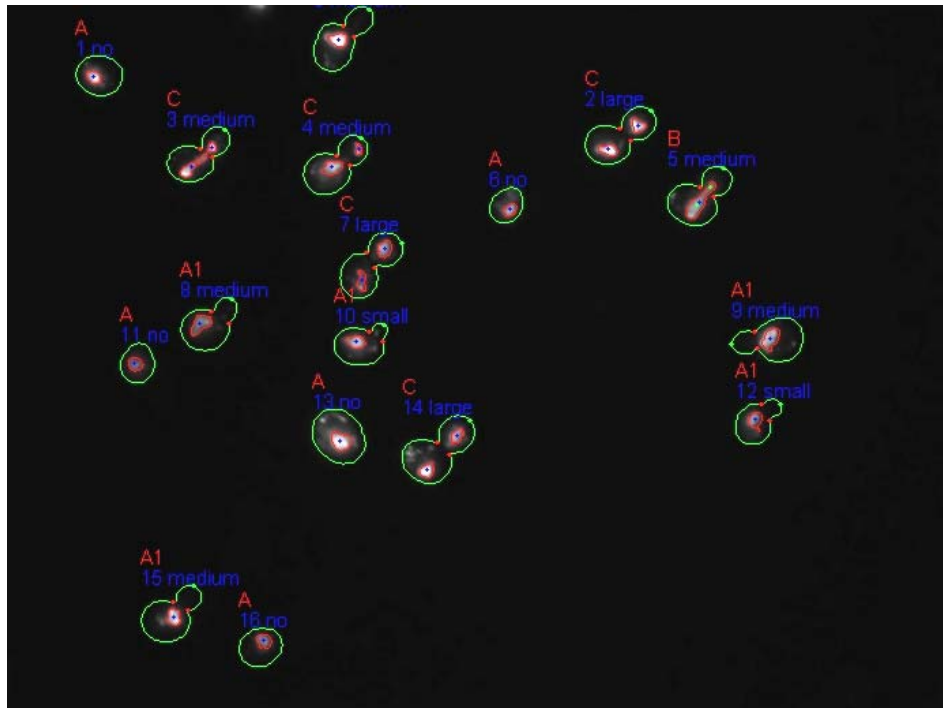


Figure 22. Example of a resulting image (Nucleus).

- Red letters: Cell cycle phase with respect to the nucleus state (Dgroup).
- Blue letters, green lines, green points, and red points: The same as in the cell wall analysis images.
- Red lines: Outline of the nucleus.
- Blue points: The center of gravity of the nucleus.
- Green points inside nucleus: Center of gravity of the part of the nucleus included in the mother cell and the center of gravity of the part included in the bud of the cells in Dgroup B.

## 5.7 Image showing the results of the actin analysis ((group name)-actin(image no.)).jpg)

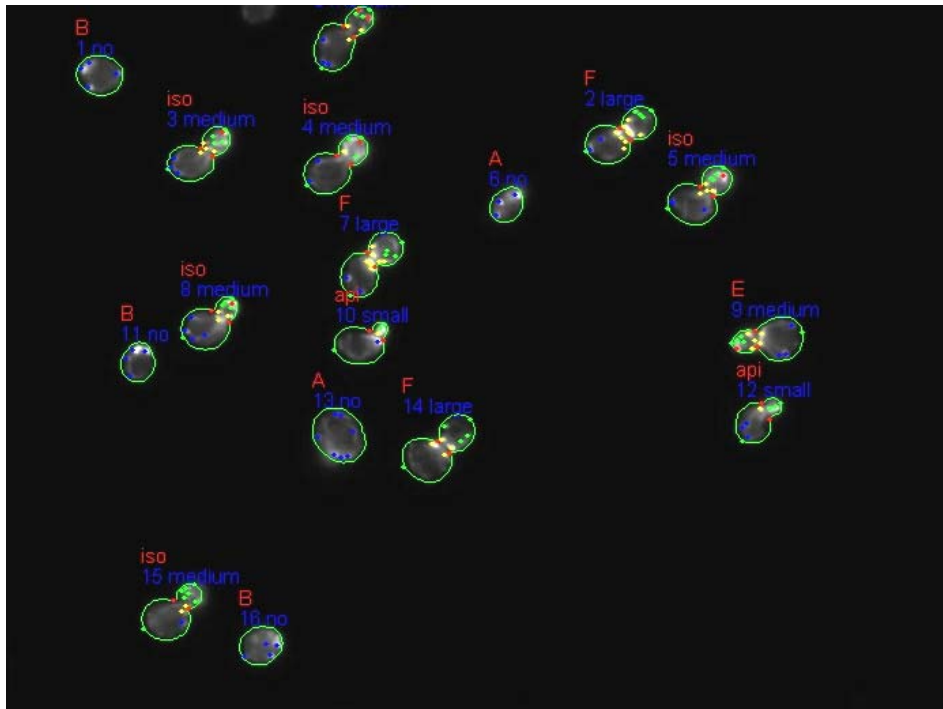


Figure 23. Example of a resulting image (Actin).

- Red letters: Cell cycle phase with respect to the actin state (Agroup)
- Blue letters, green lines, green points, and red points: The same as in the image for the cell wall analysis.
- Blue, yellow, green, and red points (inside the cells): Actin patches that were detected. The color indicates the position within the cell: In cells without buds, the patches are blue. In cells with buds, blue are patches in the mother cell far from the neck, yellow are near the neck, green are near the midpoint of the bud, and red are near the tip of the bud.

## 5.8 About exclusion\_log.txt

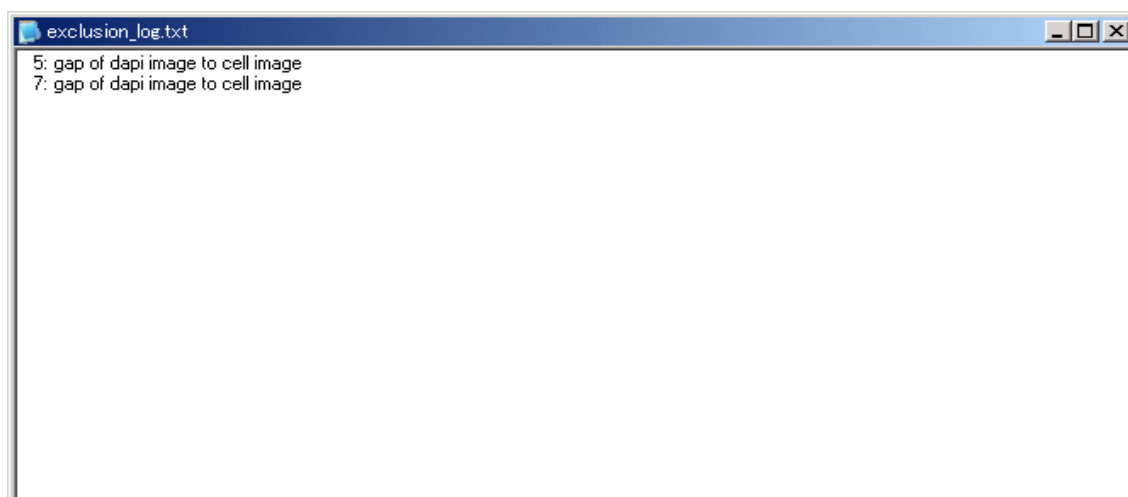


Figure 24. Example of exclusion\_log.txt

The number of an excluded image and the reason for the exclusion are recorded on the same line in exclusion\_log.txt. The reasons for exclusion are as follows:

- incorrect image size: The image size is not 696×520. Retry after size conversion.
- incorrect image color: The image is not in grayscale (256 colors). Retry after converting it to grayscale.
- gap between DAPI and cell images: The positions of individual cells in the paired nucleus and cell wall images are poorly matched, or there may be too much noise in the images.
- gap between actin and cell images: The positions of the individual cells in the paired actin and cell wall images are poorly matched, or there may be too much noise in the images.
- blurred cell image: The cell wall in the cell wall image is not clear enough to detect the cell wall position correctly.
- unexpected error: The reason is not known. The images may be extremely abnormal or there may be bugs in the software.

## **6 Culturing, staining, and observing cells ideal for use in CalMorph**

### **6.1 Cell culture and fixation**

#### *Materials*

Liquid culture medium (ex. YPD)

Cell colony grown on an agar plate

37% Formaldehyde solution, containing 8% methanol (Wako Pure Chemicals Ind. Ltd., Chuo-ku, Ohsaka, Japan, Cat. No. 064-00406)

1 M potassium phosphate buffer (K-Pi buffer, pH 6.5)

PBS buffer (TaKaRa Bio Inc., Otsu, Shiga, Japan, Cat. No. T900)

#### *Equipment*

Test tube (18 cm long, with rim) with aluminum cap

Toothpicks (or other sterilized tool for inoculation)

Pipettes

100-ml Erlenmeyer flask, closed with doubled aluminum foil

NOTE: The above equipment should be sterilized in an autoclave or by dry heat sterilization

50-ml disposable centrifuge tube

1.5-ml microtube

Rotator (TAITEC Co., Koshigaya, Saitama, Japan, RT-50)

Air incubator (TAITEC M-230F)

Water bath (TAITEC PersonalLt-10F)

Centrifuge (Hitachi Koki Co., Ltd., Minato-ku, Tokyo, Japan, himac CF7D2)

#### *Procedure*

##### Pre-culture

1. Dispense 2 ml of liquid medium into each test tube.



2. Pick up a colony from the agar plate with a toothpick, and inoculate it into the medium.
3. Culture the cells at 25°C on a rotator at 25-30 r/min.

NOTE: We usually culture the cells for about 8 h, but culture can be overnight.

### Culture and fixation

1. Dispense 20 ml of liquid medium into each 100-ml Erlenmeyer flask.
2. Transfer part of the culture to the flask, and agitate it reciprocally at 25°C in the water bath overnight.

NOTE: If possible, calculate the density of the pre-culture cells, and determine the inoculum size so that you can get a log-phase culture the next day without fail.

3. Transfer the log-phase culture ( $4 \times 10^6 \sim 1 \times 10^7$  cells/ml) to a 50-ml disposable centrifuge tube.

Add 2.5 ml of 37% formaldehyde solution and 2.5 ml 1 M K-Pi buffer.

4. Close the cap, and agitate the tube at 25°C for 30 min in a water bath.
5. Collect the cells by centrifugation (3,000 rpm, 5 min). Discard the supernatant.
6. Resuspend the cells in a mixture of 1 ml 37% formaldehyde solution, 1 ml 1 M K-Pi buffer, and 8 ml DW.
7. Incubate the tube at 25°C for 45 min.
8. Collect the cells by centrifugation. Discard the supernatant.
9. Resuspend the cells in 1 ml PBS.

NOTE: Although it is possible to store specimens at 4°C for several days at this stage, we recommend proceeding to the cell staining procedure immediately.

## **6.2 Cell Staining**

### *Materials*

PBS

P buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7.2)

FITC-ConA (Sigma-Aldrich Corp., St. Louis, MO, USA, Cat. No. C-7642)

Stock solution: 1 mg/ml in P buffer. Store it at 4°C in the dark. Long storage seems to cause uneven staining, so we usually exhaust the stock solution within one week.

Rhodamine phalloidin (Molecular Probes, R-415)

Stock solution: 200 units/ml in methanol. Store at –80°C. It keeps for several months.

DAPI (Wako, 049-18801)

Stock solution: 1 µg/ml in DW. Store at 4 °C. It keeps for about one year.

10% Triton X-100

Mounting solution: Dissolve 20 mg *p*-phenylenediamine in 1995 µl PBS and 5 µl 0.1 N NaOH in the dark. Add 18 ml glycerol (for fluorescence microscopy use: Merck Cat. No. 1.04095), and gently agitate in the dark to mix well. Dispense 400-µl aliquots into 1.5-ml disposable microtubes, and store them at –80°C. Keep in the dark, otherwise the colorless mixture will turn brown. CAUTION: *p*-phenylenediamine is carcinogenic. Avoid contact.

### *Tools*

Nail polish

Slide glass

Coverslips (22×22 mm)

Sonicator (TAITEC VR-5S)

Microcentrifuge

### *Procedure*

1. Transfer the suspended cells into 1.5-ml microtubes.
2. Collect the cells by centrifugation (3,000 rpm, 30 sec). Discard the supernatant.
3. Add 600 µl PBS. Suspend the cells.

4. Collect the cells by centrifugation (3,000 rpm, 30 sec). Discard the supernatant.

Hereafter, steps 3 to 4 are referred to as “washing”.

5. Add 90  $\mu$ l PBS, 10  $\mu$ l 200 units/ml rhodamine-phalloidin, and 1  $\mu$ l 10% Triton X-100.

Incubate at 4°C overnight in the dark.

6. Wash the cells with 600  $\mu$ l PBS.

7. Wash the cells with 600  $\mu$ l P buffer.

8. Resuspend the cells in 488  $\mu$ l P buffer and 12  $\mu$ l 1 mg/ml FITC-ConA.

9. Incubate for 5 min at room temperature.

NOTE: If the room is hot, try staining the sample for 10 min at 4°C.

NOTE 2: If the cells are stained unevenly, repeat this step.

10. Wash the cells with 600  $\mu$ l P buffer.

11. Add 600  $\mu$ l P buffer, and resuspend the cells.

12. Disperse the cells by sonication for about 5 sec at level 3 (TAITEC BR-5S).

13. Collect the cells by centrifugation, and discard the supernatant.

14. Mix 7  $\mu$ l 1  $\mu$ g/ml DAPI with 100  $\mu$ l mounting solution. Put 1.5  $\mu$ l of this on the glass slide.

15. Pick the cells up with a yellow tip; suspend them in the mounting solution on the slide glass.

NOTE: Too many cells result in areas with a high density of cells. In such cases, too many cells are classified as “complex”, and CalMorph cannot extract quantitative data. When there are very few cells, it is necessary to obtain images from many fields. Initially, try to make several specimens with different numbers of cells.

16. Put a coverslip on the drop and leave until the solution has spread sufficiently.

17. Seal the coverslip with nail polish.

NOTE: Since the cells are not fixed to the coverslip with glue, they tend to move around.

Therefore, leave the slide for at least 10 min on a flat surface in the dark before observation.

NOTE: Information on the suppliers and catalog numbers are those for the machines and chemicals that we usually use. Equivalent machines and chemicals can be used.

### **6.3 Observation and image acquisition**

#### *Equipment*

Microscope: Axioplan2 imaging (Carl Zeiss Co., Ltd., Oberkochen, Germany)

Cooled CCD camera: CoolSNAP HQ (Roper Scientific Photometrics, Tucson, AZ, USA)

Filter wheels and shutters: Systems regulated by MAC5000 (Roper Scientific)

Filter sets: FITC/DAPI/Cy3/Cy5 filter set (Roper Scientific)

Objective: PlanApo 100×oil DIC

Software for image acquisition: MetaMorph ver. 5.0 with multidimensional Acquisition/Viewer drop-in (Universal Imaging Co., Downingtown, PA, USA)

#### *Procedure*

##### Image acquisition using the “Acquire” command

First, it is necessary to confirm the settings for image acquisition.

- From the Acquire menu, choose Acquire. The Acquire dialog box will appear.

Select the Special group. Choose the settings listed below:

Sensor mode: NORMAL

Digitizer: 20 MHz (the condition for CoolSNAP-HQ: If you use another camera, set this parameter according to the manufacturer’s instructions.)

Gain: Gain 2 (4×)

Clear Count: 2

Trigger mode: Normal (TIMED)

Select the field size for image acquisition: Full chip

- From the Edit menu, choose Preferences. The Preferences dialog box will appear.

Adjust JPEG Quality to 80.

0. Create a new directory with the name of the specimen; both upper and lower case letters can be used.

1. Place a drop of immersion oil on the coverslip, and put the specimen on the stage.

2. Adjust the focus.

3. Search for a field that contains many scattered cells.

4. Acquire the image.

5. If you do not use CoolSNAP-HQ, select Image Info from the Edit menu. Check the image size. If the size is not 696×520 pixels, then the images must be trimmed using ImageMagick, Adobe Photoshop or some other image-editing software before using in CalMorph.

6. Save the image in JPEG format in the directory created at step 0. The file names should be:

Directory name-C#.jpg for FITC-ConA images

Directory name-D#.jpg for DAPI images

Directory name-A#.jpg for Rh-ph images

*Image acquisition with the MultiDimensional Acquisition command: Multiple Wavelength*

We use the “MultiDimensional Acquisition (MDA)” command to acquire images.

NOTE: the MultiDimensional Acquisition/Viewer drop-in module is necessary.

NOTE: First, it is necessary to confirm the settings for image acquisition as described in the previous chapter (*Image acquisition using the “Acquire” command*).

0. Create a new directory with the name of the specimen; both upper and lower case letters can be used.

1. From the Apps menu, choose MultiDimensional Acquisition.

2. From the Main list, select Multiple Wavelength. Select 3 for the # of waves.

3. Choose filter set 1 from Current Wavelength, and select the filter set for Rh-ph. In the same

way, select filter set 2 for FITC, and filter set 3 for DAPI. Check the filter set names with the Configure Illumination command.

NOTE: You can change the order of the filter sets.

4. Choose Select Directory, and select the directory created for the specimen. Check Increment base file name to assign the field numbers automatically.

5. Input the Base Name that you used to define the images.

6. Set the specimen, and adjust focus.

7. To set the exposure time, you can confirm the Live images, or you can use the Auto command to set an appropriate time. Repeat this procedure for each filter set.

8. Search for a field containing many scattered cells.

9. Choose Acquire.

10. If you do not use CoolSNAP-HQ, select Image Info from Edit menu. Check the image size.

If the size is not 696×520 pixels, then the images must be trimmed using ImageMagick, Adobe Photoshop or some other image-editing software before using in CalMorph.

11. Choose Save Images. The image files are saved in the directory selected at step 4.

12. Select from File, Build Stack/User Defined command. A dialog box will appear.

13. Choose the directory in which your images are saved. From the files listed, choose the files with names like “Base Name-w1filter set name.tif”, and append them into the right box.

Select OK. This procedure arranges a series of TIFF files into a stack file. Make stack files for the files for the other dyes.

NOTE: Ensure that the numbers are the same for images of the same field stained with different dyes.

14. From the File menu, select Save as... Save the files in JPEG format. Name them “directory name-C.jpg”, “directory name-A.jpg”, and “directory name-D.jpg”. Field numbers are automatically applied in order.

15. Discard the nd files, TIFF files, and thumbnail files from the directory.

If you are using an automatic XYZ stage, choose Multiple Positions from the MDA Main menu.

Avoid acquiring repeated images from the same fields. The files have names like “Base Name-w1filter set name-s1.tif”. “s1” means “stage position 1”. Change the file names as described in steps 13 and 14 of Image acquisition with the MultiDimensional Acquisition command: Multiple wavelength. Discard the nd files, TIFF files, and thumbnail files from the directory.

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