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Summary

SINGLE CELL ANALYSIS OF YEAST COLONIES IN MITOTIC ARREST ADAPTATION

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Summary

This thesis work is part of a line of research on the study of the control system of the cell division cycle.

Each cell is derived, in fact, from its progenitor cell, hence the importance and sensitivity of the process of cell replication.

The cell cycle can be divided into two phases: a synthesis (**S** phase) of the genetic material through the copying of DNA and the physical link of the duplicated chromosomes (*sister chromatids*), and a known mitotic (**M** phase), which has the nuclear division (*mitosis*) and, finally, cellular division (*cytokinesis*).

The cell cycle progresses thanks to a family of enzymes called cyclin-dependent kinases (*Cdks*) that are active only when they are related to particular proteins called cyclins, which alternate in time articulating the different stages of cell replication.

The activity of these kinases is controlled by surveillance mechanisms called **checkpoints**.

In this thesis project have been taken care of one of these control points, called the *Spindle Assembly Checkpoint* (**SAC**), which verifies the correctness of the separation of sister chromatids (*chromosome segregation*) and stops cell division in case of problems.

In particular, we have dealt with the phenomenon of **adaptation to the checkpoint** for which a cell arrested in mitosis by the SAC fails to replicate anyway.

The phenomenon of adaptation is relevant as it is observed in the case of cells undergoing treatment with antimitotic drugs.

The Spindle Assembly Checkpoint

The SAC monitors the transition from *metaphase*, when the chromosomes are aligned, forming the so-called *metaphase plate*, to *anaphase*, in which the sister chromatids are separated by structures called *microtubules* that pull them towards the two opposite poles of the cell.

This transition can be divided into two events: the separation of chromatids, through the degradation of *cohesin* (the protein complex that holds sister chromatids

physically linked) and the mitotic cyclin degradation. Both of these events are catalyzed by a protein complex called APC (*Anaphase Promoting Complex*), which is active only when bound to the protein Cdc20 (*Cell Division Cycle Protein 20*). The SAC is part of this transition by monitoring the proper attachment of microtubules to the chromosomes. Following an incorrect, or missing, coupling between microtubules and chromosomes the checkpoint leads to sequestration of the protein Cdc20 in another protein complex called MCC (*Mitotic Checkpoint Complex*), inhibiting the activity of the APC and, ultimately, not allowing entry into anaphase.

The importance of this surveillance mechanism resides in the fact that an error in this phase of the cell cycle causes a duplication with unequal number of chromosomes (aneuploidy) of the two cells generated.
The aneuploidy is not necessarily lethal, but is highly correlated to pathologies such as tumors and other genetic diseases (for example, Down syndrome or *trisomy21*). For this evolution has created a surveillance mechanism, the SAC, to avoid it. As previously mentioned, many anticancer drugs, in clinical use, using the SAC to stop the growth of cancer cells, destabilizing the bond with the microtubules. Cells subjected to a prolonged stimulus activating the checkpoint in the end adapt, and continue in cell division, although unable to properly divide the DNA.
In this thesis, we want, therefore, to investigate the phenomenon of adaptation by studying their correlation with the two proteins more involved in the metaphase-anaphase transition (Cdc20 and mitotic cyclin CLB2) and also trying to understand the behavior of cells that are unable to replicate in the presence of a stop signal to the checkpoint.

Experimental Techniques

To study the Spindle Assembly Checkpoint in this thesis we used the model organism *Saccharomyces cerevisiae*, a unicellular eukaryote yeast, because it is easily manipulated genetically, has a short doubling time and shares the molecular network with the most advanced multicellular eukaryotes.

Being the adaptation phenomenon **highly variable**, it was necessary to turn to a

technique able to capture the behavior of each single cell, known as ***single cell analyisis***. This technique involves the use of time-lapse fluorescence microscopy of cell colonies.

Through the collection of multiple images, at regular time intervals, it is possible to capture the behavior in time of the individual cells of a yeast colony under specific conditions, such as those of the adaptation.

The availability of naturally **fluorescent proteins** (*GFP, mCherry, YFP*) makes it possible, through a process called *tagging*, the labeling of proteins of interest. It is, in fact, possible to fuse the gene sequence that encodes a protein in a gene sequence coding for the fluorescent protein.

In this way it was possible to use yeast strains that produced proteins of interest (Cdc20 and CLB2) fluorescent, thus linking the emitted fluorescence with the concentration of the protein.

The use of a microscope capable of acquiring sequences of frames resulting from the fluorescent emissions, thus has allowed to detect the dynamics of proteins of interest in the presence of the Spindle Assembly Checkpoint constantly induced. The activation of the SAC was induced by the use of strains where the presence of galactose in the cell culture medium induced over-expression of a protein of the checkpoint, Mad2. The latter is part of the MCC that sequesters Cdc20, and therefore its over-expression does not allow the activation of the APC and the passage in anaphase even if the link between microtubules and kinetochores normally occurs. It is a SAC comparable to that generated by drugs, such as *nocodazole*, which acts directly on microtubules, permitting the adaptation and, in addition, is inducible at will by changing the cell culture medium.

The experimental instrumentation is completed by the presence of microfluidic plates for *live cell imaging* through which it is possible to grow cells. Due to the long duration of the experiments, necessary for the study of adaptation, they are used of the microfluidic chambers which allow, by perfusion, the maintenance of the nutriment of the cells.

The used chambers also address the problem of vertical overlapping of the cells,

through a system of trapping along the horizontal plane, and this is very important because it allows the recognition of a single cell in an image.

Image Analysis

After the acquisition of the frame sequence, through a digital camera attached to the microscope, there is one of the basic step in single cell analysis: **the image analysis**. The basic steps that must carry out an analysis software of images in single cell analysis are three: identification of every single cellular body, its contours extraction (**segmentation**) and ability to follow in the various frames of the sequence the same cellular body.

There are several software that deal with this, among which it was decided to use *Phylocell*, a MATLAB code used for the study of colonies of Saccharomyces cerevisiae under conditions not disturbed, that is favorable environment for cell growth.

The choice fell on this program as the most accurate in segmenting the yeast cells. At the same time, *Phylocell* has several limitations, which have been addressed in the thesis. The first limit is evident in terms of checkpoints induced in the experiments. The arrest of cell division induced by the checkpoint causes a **morphological change** in the cells: rounded in non-perturbed and non-circular in adaptation. This morphological difference is reflected in incorrect segmentations by *Phylocell*, which in its segmentation strategy utilizes strong geometric assumptions of the cells, the work of this thesis was, therefore, also to solve this difficulty.

The proposed solution involves the use of a segmentation *Region Based* strategy, which does not consider the morphology of the object to be extracted, which takes the name of **Marker Controlled Watershed**.

This method interprets the image as a topographic surface with mountains and valleys where the height of the surface is determined by the intensity of the pixel, extracting contours as the lines representing the watershed, imagining fill with water (which will flow towards the closer minimum topographic) the entire region of the image.

The use of markers, regions of the image where is the value is imposed at the

minimum, solves one of the main problems of the strategy Watershed: the over-segmentation, we have used the contours extracted from Phylocell for identifying these regions of minimum.

Another criticality of *Phylocell* highlighted by our analyzes is the segmentation of the beginning of the **budding phase**, inasmuch the geometric parameters used by this software include the use of an average diameter that does not allow the extraction of contours of objects too small (such as the bud of the yeast).

Also to solve this type of error is proposed a *Region Based* segmentation strategy, which does not consider the morphology of the object to be extracted, called **Region Growing**, which use a criterion of homogeneity (in the case of bud recovery is the intensity) of the neighboring pixels to a starting pixel (seed) to merge them into a single region. The method implemented in addition to the criterion of homogeneity, also uses a criterion of maximum distance from the starting point and threshold levels of intensity minimum and maximum to extract the contours.

Also for this method are used the contours obtained from Phylocell as a starting point: the seed for the segmentation Region Growing is identified as the centroid of the segmentation to the next frame and, also, for the detection of the intensity threshold.

These two methods, the budding phase recovery and not circular cell recovery, have been implemented in the graphical user interface of Phylocell, inserting two additional graphical tools to solve two problems (wrong seed for the recovery and identification of the phase of budding as a single body cell of two cell extraction with Watershed) very experienced in their use: a tool for manual reseeding in the bud recovery and a tool for automatic division along the bottleneck of a unique cellular body in the non-circular cell recovery.

To verify the quality of the implemented methods were compared with the manual segmentations, taken as a reference. These tests have given good results in terms of number of errors recovered and of overlap with the contours handmade.

Biological Result

The instrumentation described and the methods of image analysis introduced were used for the single cell analysis of three experiments: i) correlation between adaptation and the mitotic cyclin Clb2 (required to activate the APC and then to enter anaphase), ii) correlation between adaptation and activator of APC Cdc20 and iii) study of adaptation memory.

The study of mitotic cyclin has highlighted the absence of any amount of threshold at which the cells adapt, and thus a **lack of direct correlation between Clb2 and adaptation** to the checkpoint. Regarding the dynamics of the mitotic cyclin it is seen that the levels of this protein will accumulate under conditions of SAC active up to a plateau level for a long period, then once divided degrade faster: this is in contrast with the results obtained by studies in the population (e.g. Western Blot) in which erroneously deduced a slow and steady degradation of Clb2.

The study of Cdc20, because of its rapid degradation that does not allow the maturation of the fluorophore and thus does not allow to see the signal in fluorescence, was done in an indirect way. It was decided to look for a correlation between the synthesis of Cdc20 and adaptation to see the synthesis, using a fluorophore product exactly as Cdc20. The image analysis showed no differences between the two populations and therefore we concluded that the synthesis of **Cdc20 does not drive the phenomenon of adaptation**.

Then we wanted to investigate the role of this protein by decreasing its degradation. For this we used strains without a protein (*Mnd2*) involved in the degradation of Cdc20. It is shown that in strains with the deleted Mnd2 (and therefore with a greater amount of Cdc20) the phenomenon of adaptation occurs faster and more synchronous, suggesting an **involvement of this protein in adaptation to the mitotic checkpoint**.

Studying what happens after the adaptation we sensed the presence of a **form of memory, or loss of sensitivity, to the SAC** which shortens the duration of cell cycles after a first cycle with a long adaptation time.