Constructing and testing of reporters of the cell cycle

Deliverable 6.1 [DEM,PU]

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1. Introduction

The main objectives of WP6 is to demonstrate that both external and embedded control of gene expression can be used to drive the cell cycle in yeast with the ultimate aim of obtaining a (self) synchronizing yeast population endowed with the property that all the cells within the population bud at the same time. All the available methods in yeast biology do not really "synchronise" the cell population, at least according to the terminology of dynamical system theory, but rather just force each cell in the population to be at the same initial condition. The first task, to which the deliverable D6.1 is linked, aimed at ensuring that we could both monitor and act on the cell cycle by designing new strains or collecting strains from the literature and testing them.

1.1. Experimental Platform for yeast strain characterisation.

To quantitatively analyse and perturb the cell-cycle in the yeast strains, we made use of automated microfluidics feedback control of gene expression to precisely regulate the amount of α-synuclein

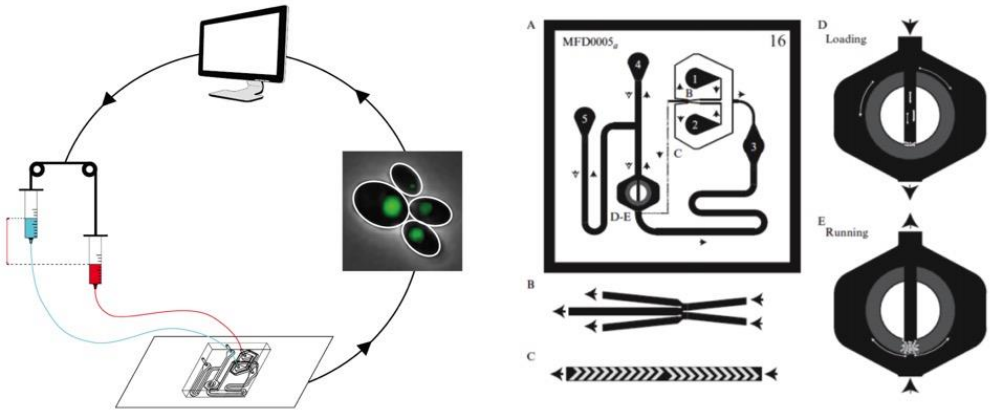

Figure 1: Automated microfluidics feedback platform*. The platform (left) consists of a microfluidic device, a time lapse microscopy and a set of automated syringes, all controlled by a computer. Cells grow in a microfluidic device (right) within a temperature-controlled environment under an inverted fluorescence microscope. Images are acquired at regular sampling time, and quantification of the fluorescence is performed via an image segmentation algorithm.*

produced by the cells over time, as shown in **Figure 1** (Fiore et al. 2016; Menolascina et al. 2014). Briefly, this automated platform performs the following steps: (1) imaging of yeast cells within the microfludics device with a time-lapse fluorescence microscope at 2 min sampling intervals; (2) quantification of

fluorescence levels of one or more reporters from fluorescent images with a custom image processing algorithm; (3) computer-based control of two automated syringes to deliver one of two different growth medium to the cell chamber in the microfluidic device.

1.2. Yeast strains enabling cell cycle control

To act on the cell cycle, following the work of (Charvin, Cross, and Siggia 2009), we aim at controlling the level of expression of the cyclin CLN2, a key actor expressed periodically by the cell in order to overcome the cell cycle checkpoints and initiate the S phase. We identified and collected a number of yeast strains that have been reported in the literature and that are listed in Table 1 (Charvin, Cross, and Siggia 2009) (Rahi et al. 2016, 2017). These strains can be divided in two categories, as shown in Figure 1: the cycling strains, which are able to cycle also in the absence of CLN2 expression, and the non-cycling strains, which require CLN2 expression to initiate cell-cycle and budding.

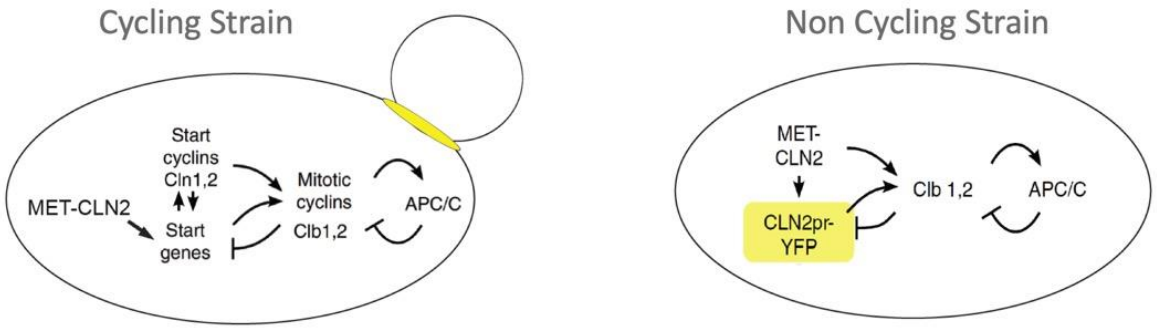

Figure 2: Yeast strains with inducible CLN2 expression*. The strains reported in the literature can be subdivided in two broad categories: the cycling strain (left) does not require CLN2 expression to initiate the cell cycle and the subsequent budding, unlike the non-cycling strain (right). The cycling strain uses as reporter the CDC10-YFP protein that stains the bud neck of budded cells. The non-cycling strains uses as reporter the YFP under the endogeneous CLN2 promoter.*

These strains enable to change the level of expression of CLN2 (and of additional cyclins of interest such as CLB1) by changing the growth medium within a microfluidic device (e.g. addition or removal of methionine, glucose, galactose). Note that the initial idea of this deliverable was to design a double fluorescent reporter for the cell-cycle. However, after testing the strains we obtained with inducible CLN2 expression, we decided it was not practical to build such a complex reporter, as it would have required to redesign the strains, because of the limited selection markers available. Moreover, adding two fluorescent reporters requires additional constrains when implementing light activation of gene expression by optogenetic for single-cell control, a key challenge of Work-Package 6. Instead, we decided

to improve the image segmentation and tracking software to monitor the cell cycle progression quantitatively at the single cell level using the available fluorescence reporters together with quantitative analysis of the yeast morphology (Lugagne et al. 2018; Fiore et al. 2016).

1.3. Monitoring cell-cycle phase in the S1 cycling strain.

In the S1 cycling strain, it is possible to monitor yeast bud-neck morphology as well as its initiation with the fluorescently tagged CDC10 bud-neck marker (Fig. 1). This marker has been shown to be a very robust indicator of the appearance of the bud-neck, and thus of the G1/S phase transition of the cell-cycle.

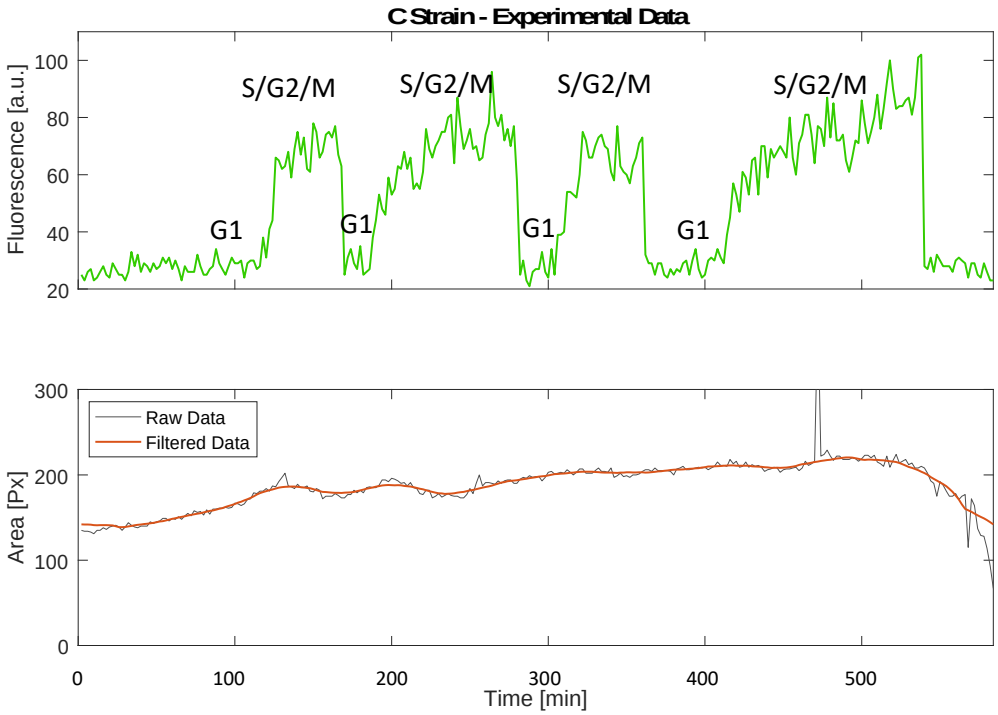

Figure 3: Experimental characterisation of the S1 cycling strain. The top panel reports the fluorescence level in a single cell measured from microscopic images taken at 2 min interval of the CDC10-YFP. In the lower panel the area of the same is reported using pixels as unit of measure. Cells were grown in the presence of methionine.

Another marker of the cell cycle available in the cycling strains is the Whi5-GFP. We performed an initial characterisation of the S1 strain, as shown in Fig. 2, by growing cells in the presence of methionine. We then adapted a software for image segmentation and tracking in order to detect bud-neck formation and to quantify the area of the cell (Fiore et al. 2016; Menolascina et al. 2014)(Lugagne et al. 2018). The G1 phase can thus be distinguished from the S/G2/M phases thanks to the CDC10-YFP fluorescence level. However, it is not possible to distinguish among the S/G2/M phases in this strain.

1.4. Monitoring cell-cycle phase in the S4 non-cycling strain.

The S4 strain has two fluorescence reporters: a nuclear mCherry and a Venus expressed from the endogenous CLN2 promoter. This strain is not able to cycle in the presence of methionine, as the G1 cyclin CLN2 is expressed from a methionine repressible promoter and all the three cyclins (CLN1, 2 and 3) are deleted in this strain. As the CLN2 promoter is activated in G1 and repressed in G2, the Venus fluorescence could in principle be used to track the cell-cycle. However, we observed that even in the presence of methionine, which causes a block in the G1 phase of the cell cycle thus preventing budding, expression of the Venus protein from the endogenous CLN2 promoter may still occur and hence by itself Venus fluorescence is not a robust marker of the cell cycle. Therefore, to improve the cell-cycle phase estimation, we considered also the cell volume. Indeed, as demonstrated in (Charvin, et al., 2009), cell volume increases only during G_1 phase, while in the other phases, the increase in the cell mass is used for the bud growth. Thus, we decided to infer the cell cycle phase θ in this way:

$$\theta = \begin{cases} 0 & \dot{V} > 0 \\ \theta_{CLN2\,pr} & \dot{V} \le 0 \end{cases} \qquad (1)$$

During volume growth, we set the cell cycle phase at 0 (i.e. the beginning of G_1 phase), otherwise we infer the cell cycle phase from the fluorescence of Venus protein.

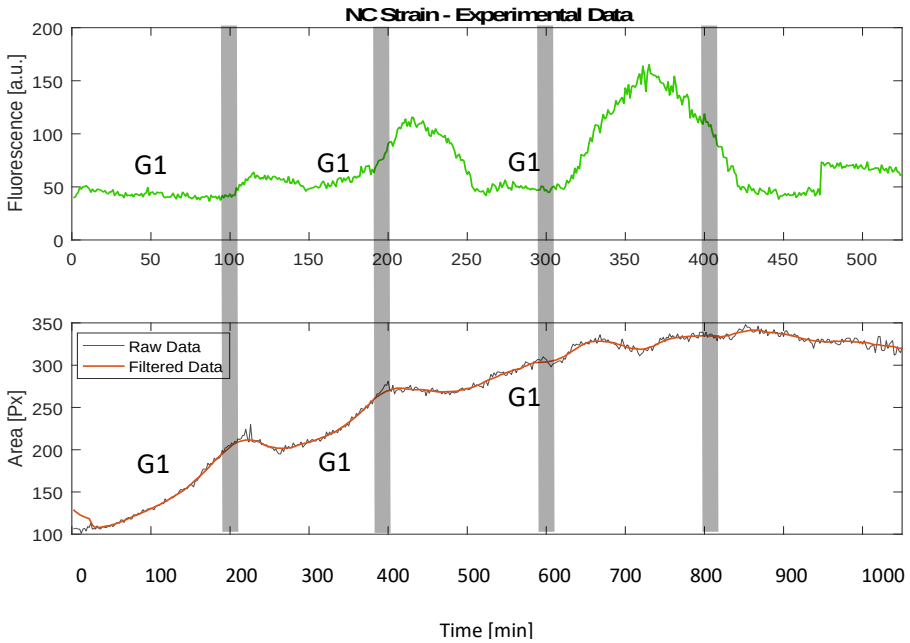

Figure 4: Estimation of the cell cycle phase. At time 0 methionine is added to the medium thus causing cells to stop in G1. At 180 min interval, methionine is removed for 30 min (grey bars), allowing CLN2 expression. The top panel reports the quantification of the Venus reporter fluorescence expressed from the CLN2 promoter in a single cell. The bottom panel is the area in pixels of the same cell.

In order to estimate the volume of the cell, the image segmentation algorithm was modified to estimate the area of the cell in pixels. An example of Venus fluorescence quantification and area estimation in a single cell is shown in Fig. 4. The experiment starts with methionine-rich medium, and the at 180 min intervals, medium is switched to a no-methionine medium for 30 min (grey bars in Fig.4) and then switched back to methionine-rich medium. Notice that at 810 min, despite the switch to no-methionine medium, the cell does not bud as the volume has grown too much and the cell is not able to start a new cycle. This happens as the natural period of this yeast strain when grown in the absence of methionine is about 80 min, whereas we forced the cell cycle to be 210 min (180 min+ 30 min) thus decoupling cell volume growth from cell division and causing the cells to grow too much.

Table 1. List of Yeast strains available in COSY-BIO.

ID	YEAST STRAIN
S1	MAT a cln3::LEU2 CDC10-YFP-LEU2 WHI5-GFP-KanMx TRP1::Met-CLN2
S2	MAT a cln3::LEU2 CDC10-YFP::LEU2 ADE2
S3	MAT α CDC10-YFP-LEU2 WHI5-GFP-KanMx TRP1::Met-CLN2 ADE2
S4	cln1Δ cln2Δ:CLN2pr-Venus:TRP1 cln3Δ:LEU2 trp1Δ:TRP1:MET3-CLN2 HTB2-mCherry:HIS5
S5	cln1Δ cln2Δ:CLN2pr-Venus:TRP1 cln3Δ:LEU2 trp1Δ:TRP1:MET3-CLN2 clb1Δ-clb6Δ:KanMX clb2Δ:GALL-CLB2:URA3-clb5Δ:KanMX clb3Δ:TRP1 clb4Δ:his3:KanMX HTB2-mCherry:HIS5
S6	cln1Δ cln2Δ cln3Δ:LEU2 trp1Δ:TRP1:MET3-CLN2 SIC1:SIC1pr-YFP:URA3
S7	cln1Δ cln2Δ cln3Δ:LEU2 trp1Δ:TRP1:MET3-CLN2 clb1Δ-clb6Δ:KanMX clb2Δ:GALL-CLB2:URA3-clb5Δ:KanMX clb3Δ:TRP1 HTB2-mCherry:HIS5 SIC1:SIC1pr-YFP:URA3
S8	cln1Δ cln2Δ cln3Δ:LEU2 trp1Δ:TRP1:MET3-CLN2 CLB2:CLB2pr-GFP:NatMX
S9	cln1Δ cln2Δ cln3Δ:LEU2 trp1Δ:TRP1:MET3-CLN2 clb1Δ-clb6Δ:KanMX clb2Δ:GALL-CLB2:URA3-clb5Δ:KanMX clb3Δ:TRP1 HTB2-mCherry:HIS5 CLB2:CLB2pr-GFP:NatMX

1.5. Conclusion

We have now a comprehensive list of strains that have been tested and that can be used to activate the expression of cyclins by changing growth medium. We have also improved the image segmentation algorithm to automatically identify bud-neck formation, quantify the cell size and the fluorescence from the CLN2 promoter, thus enabling cell-cycle phase determination. Both the software and the strains are available on request.

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Website links:

COSYBIO link project website: http://www.cosy-bio.eu/