The mother enrichment program; a way to study ageing in yeast

Bachelorthesis
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Abstract

A mother enrichment program in *Saccharomyces cerevisiae* was investigated. In the strain 5185 it was found that upon adding 20 μM estradiol the daughter cells were unable to divide giving rise to slower growth of the culture and therefore a higher fraction of (old) mother cells. However, the program was not working with a 100% efficiency. An increase of the estradiol concentration did not show any effect.

Contents

Introduction

Ageing is a process which is not yet understood. One approach to learn more about this process is to observe a cell while it ages and look what changes. For this we should be able to look at old cells. Unfortunately, old cells get overgrown by their daughters, grand-daughters and great-grand-daughters very fast. In this bachelor project we investigated the mother enrichment program, a program with which, hopefully, we will be able to study old cells exclusively. We tried to standardize a procedure to get old mothers, without a lot of younger cells. Hopefully others can use this procedure to study the complicated process of ageing.

Growth of yeast

In this section a short introduction will be given into the growth of yeast. S*accharomyces cerevisiae* (budding yeast) divides by budding, which means a bud is formed on the surface of the mother cell, which eventually seperates from the mother cell, giving rise to a small daughter cell. It divides asymetrically, therefore giving a large mother cell and a smaller daughter cell. At the bud neck, an age barrier prevents ageing factors, such as accumulated proteins, extrachromosomal ribosomal DNA circles (ERC's) and oxidatively damaged proteins to flow to the daughter cell. The daughter cell, therefore, can begin it's life (relatively) free of age associated damage. Every time a daughter cell is formed, a bud scar forms on the mother cell. It is possible to count these bud scars and determine how many divisions a mother cell has completed.

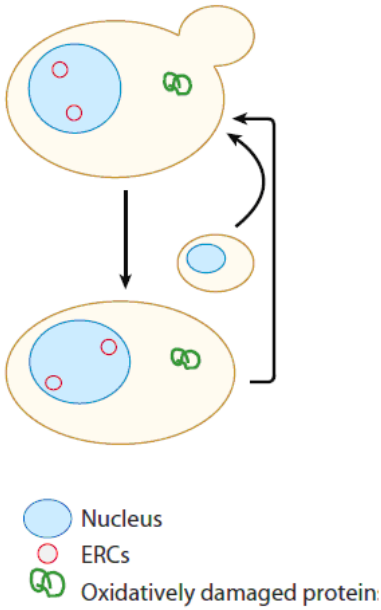

Figure 1. The growth of a yeast cell [1]

The membrane of the bud (and thus the new cell) is formed from new material. Therefore when the membrane of a cell is stained, it will not pass the dye to the membrane of her daughter cell. Because of this, once the cells in a culture are stained, it is possible to distinguish old cells (formed before the staining) from new cells (formed after the staining).

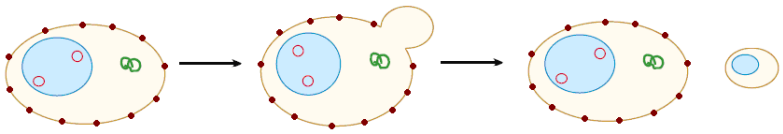

Figure 2. When a stained yeast cell produces a daughter, the dye will stay in the mother's membrane, giving rise to a non-fluorescent daughter

When yeast is grown in an excess of glucose, it rapidly grows, generating ethanol as a major by-product. The cell in this conditions divides every two hours. When glucose is exhausted the cells will stop dividing for several hours. The metabolic system then shifts to the use of ethanol. This shift is called the diauxic shift. After this they will continue growing, although slower (generation time 5-6 hours in minimal medium), consuming ethanol. After a few more divisions the cell density get's so high that essential nutrients are exhausted. The yeast cells then stop dividing and enter the so called stationary phase (Stat-phase).[2]

What is ageing?

Ageing in yeast can be described as chronological ageing (the capability of cells to maintain viability over time) or replicative life span (the number of times a cell can divide before it undergoes senescence). [3] In this report we look mainly to replicative life span (RLS) of yeast cells. Ageing is described as a los of function, an increase in mortality and a decrease in fertility. But what is it that is actually causing this process? Several studies led to the idea of a "senescence factor"; something that accumulates in cells during their life and limits their life span. Here some candidates for this senescence factor will be discussed.

ERC's as an ageing factor
One candidate for the search for senescence factors in budding yeast are extrachromosomal DNA circles (ERC's). Recombination can generate these ERC's. Thereafter they can be replicated. When the mother cell divides it keeps these ERC's to herself, thereby "resetting" the daughter cell to zero. It is shown that high levels of ERC's limits the life span in budding yeast, whereas limiting the generation of ERC's extends life span, supporting the idea they might be a senescence factor. However, there are also results which suggests that ERC's are not the only senescence factor. Because of this and the fact that there is no evidence for ERC's as ageing factor in organisms other than budding yeast, it is suggested that we might have to look for other causes for ageing as well. [4]

Oxidatively damaged proteins as an ageing factor
Another species that is proposed to be a senescence factor are oxidatively damaged proteins. Unlike ERC's, oxidative damage is correlated with ageing in many organisms. Although there is some evidence that these damaged proteins might act as a senescence factor, this evidence is not yet convincing. Therefore they will have to be studied more carefully.

Protein aggregates as an ageing factor
The last possibility discussed here is that of protein aggregates as a cause for ageing. The oxidatively damaged proteins mentioned before can form aggregates. However, as for the oxidatively damaged proteins, there is no convincing evidence so far that these aggregates act as a senescence factor. [5]

In this thesis there will be no answer to the question what really causes ageing. A method, however, is provided to investigate this in future researches.

The Mother Enrichment Program (MEP)

One of the biggest problems in the research of the ageing of yeast cells is that it's very difficult to follow an ageing cell in a growing population of yeast, since the culture grows exponentially. As can be seen in figure 3, even after four generations, there is just one old cell on every 15 younger cells. In higher generations there will be even a smaller fraction of mother cells. Since an old cell is at least 15 generations old, there will only be a negligible amount of mother cells in the culture.

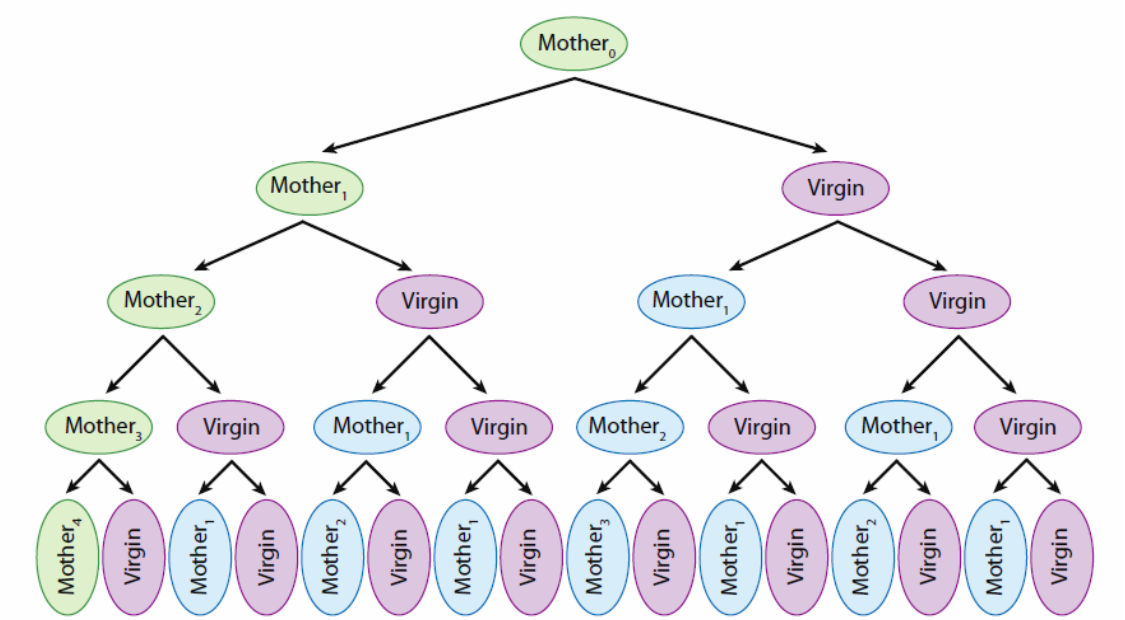

Figure 3. Four generations of growth in a "normal" yeast culture [1]

When we measure the growth of this culture the amount of cells will increase exponentially and therefore the optical density (which is proportional to the amount of cells) will also increase exponentially. This was measured and the growth curve produced from this is shown in figure 4.

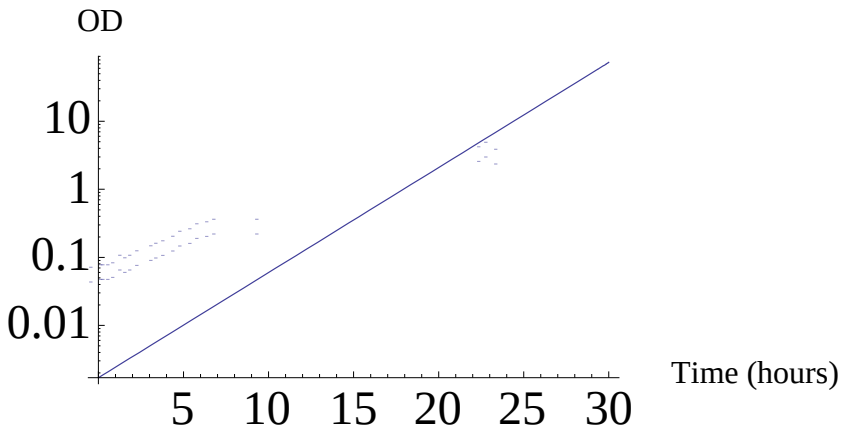

Figure 4. Growth curve of a "normal" yeast culture

To be able to study these old cells, to see what changes when they age, we want to minimize the amount of younger cells. Since we want the mother to maintain an normal replicative life span, we want this cell to divide. However, we do not want the daughter to divide, since that will cause an exponentially growing culture. Therefore we would like to have a culture, as shown in figure 5. To accomplish this we use the mother enrichment program (MEP). This is a genetic construct which eliminates the proliferative potential in daughter cells while it allows the mother cells to maintain a normal replicative life span. This construct uses a Cre-Lox inducible combination to disrupt two genes, UBC9 and CDC20 in daughter cells. Both genes are essential for yeast because they are required for the degradation of targets vital to cell cycle progression. When these genes are eliminated in daughter-cells, they will permanently arrest in M-phase, which means they are unable to divide (no new daughter cells will be produced by this cell). The Cre recombinase is only expressed in daughter cells because a daughter-specific promoter derived from SCW11 is used. The activity of the cre-recombinase is also regulated by estradiol. When estradiol is added the recombinase is transported into the nucleus and can there act upon it's targets. With other words, the MEP provides a way to selectively limit the proliferation of daughter cells upon addition of estradiol, while the mother cells maintain a normal replicative life span. [3]

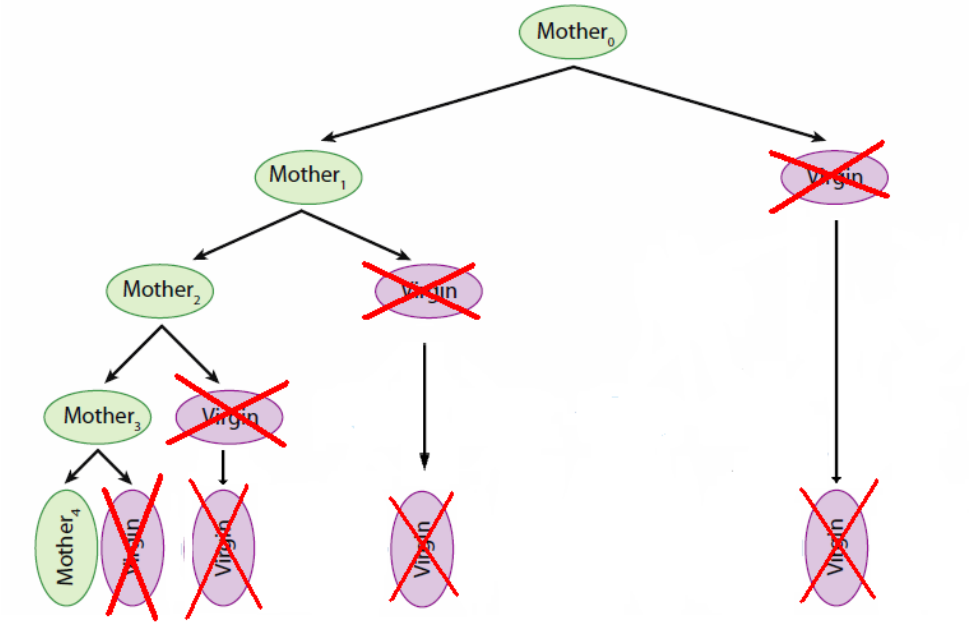

Figure 5. Four generations of growth in a yeast culture when the MEP is switched on

Once we use the mother enrichment program, we would thus like the growth curve to be linear and look like the orange curve in figure 6.

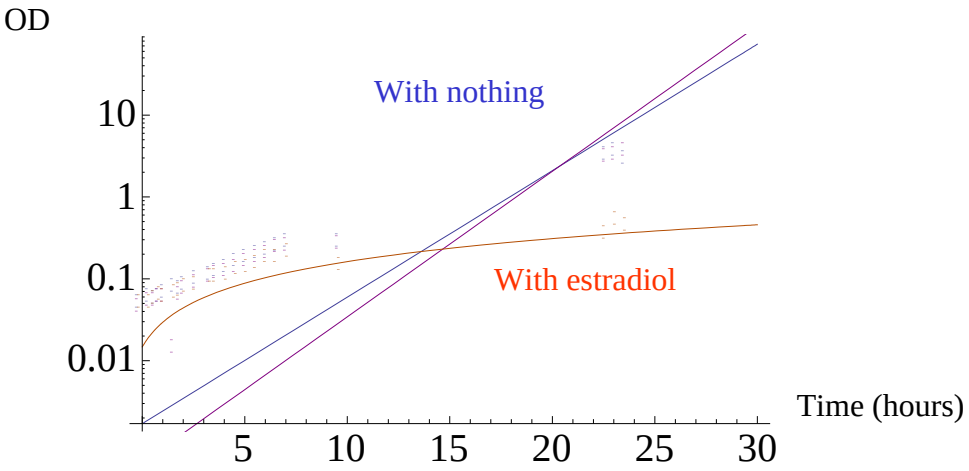

Figure 6. Growth curve of yeast cultures (UCC 5185) with the MEP with and without estradiol

There are a few more things to take into consideration when using the MEP. First, although daughter cells will arrest in M-phase, they can still be metabolically active for ~24 hr. Therefore the mother cell still has to be separated from the daughter cells. Second, random mutations can occur that inactivate the MEP and therefore produce proliferating daughter cells. [3]

Materials and Method

Strains
The strains used in this study are:
UCC5179 (MAT**a** ade2::hisG his3 leu2 lys2 ura3 Δ0 trp1 Δ63 ho Δ:: P_{SCW11}-*cre-EBD78-NATMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX*),

UCC5181 (MATα ade2::hisG his3 leu2 trp1 Δ63 ura3 Δ0 met15 Δ::ADE2 *ho* Δ *::*P_{SCW11}*-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2 loxP-CDC20-Intron-loxP-HPHMX*),

UCC5185 (MAT**a**/MATα ade2::hisGhis3/his3 leu2/leu2 LYS2/lys2 uxa3Δ0/ura3Δ0 trp1 Δ *63/trp1* Δ *63 MET15/met15* Δ *::ADE2 ho* Δ *::*P_{SCW11}*-cre-EBD78-NATMX/ho* Δ *::*P_{SCW11}*-cre-EBD78-NATMX loxP-UBC9-loxP-LEU2/ loxP-UBC9-loxP-LEU2loxP-CDC20-Intron-loxP-HPHMX/loxP-CDC20-Intron-loxP-HPMX)*

Cultivation
The strains were grown at 30ºC in an incubator. The cells were grown in minimal medium with additional complete supplement and adenine sulphate.

Cell staining
The cells were stained using a red fluorescent cell linker kit for general membrane labeling (PKH26GL-1KT).
To stain the cells first the culture was centrifuged. Then the pellet was resuspended in a small amount of medium and was added to 500µL dilute solution. The solution was shaked for three minutes. Then the solution was added to a solution of 20µL stain in 480 µL dilute solution. This was shaked for three minutes. The solution was added to 4,5 ml BSA-solution and was then centrifuged. The pellet was suspended in a small amount of medium and then added to the medium to start a culture.

Flow cytometer
A flow cytometer was used to look with more detail to the cultures. For every measurement 50,000 events were measured. The flow rate was set to 35µL per minute and the core size was 16 µm. No threshold was used.

Glucose measurements
The concentration of glucose was measured during time, using a Glucose (HK) Assay Kit. The cultures were diluted 10 times and were centrifuged. 30 µL of the supernatant was put in a cuvet with 170 µL of the Glucose Estimation reagent. After 15 minutes 800µL demiwater was added. The OD was then measured at 340 nm with the photospectrometer.

Results

First we wanted to know whether the MEP in the yeast cultures was working. Therefore we made growth curves of the cultures with and without estradiol. Then we found that it was working in the 5185 strain (figure 7). In the other cultures it was not working very well but it remains unclear whether this is due to the low concentration of estradiol, or the MEP is not working in these strains.

Next we wanted to know whether the slower growth of the culture was really due to the estradiol and not to the ethanol in which it was dissolved. Therefore we also made a growth curve of a culture with ethanol, but without estradiol. We found that the ethanol did not influence the growth of the culture, as can be seen in figure 7.

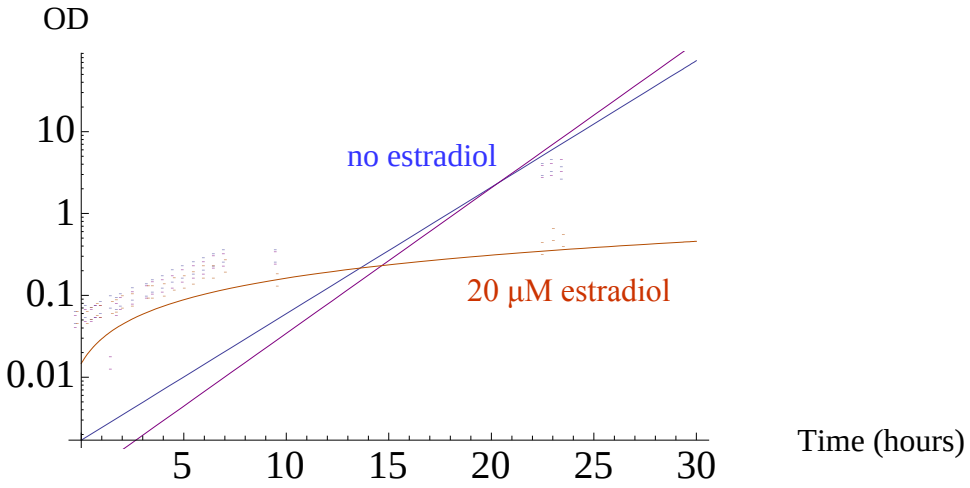

Figure 7. Growth curve 5185 with nothing (blue), ethanol (purple), 20 µM estradiol (orange) and 200 (red) µM estradiol

Now we wanted to look with more detail to the growth of the culture, and see what happened to the amount of mother cells and daughter cells during time. Therefore we stained the cells (the mother cells will stay fluorescent, whereas the daughters will not be fluorescent) and looked at them with the flow cytometer. Then we found that, although the fraction of mothers was decreasing a lot slower in the cultures with estradiol, then in the cultures without estradiol, it was still not decreasing linearly and can therefore still be improved (Figure 8 and 9).

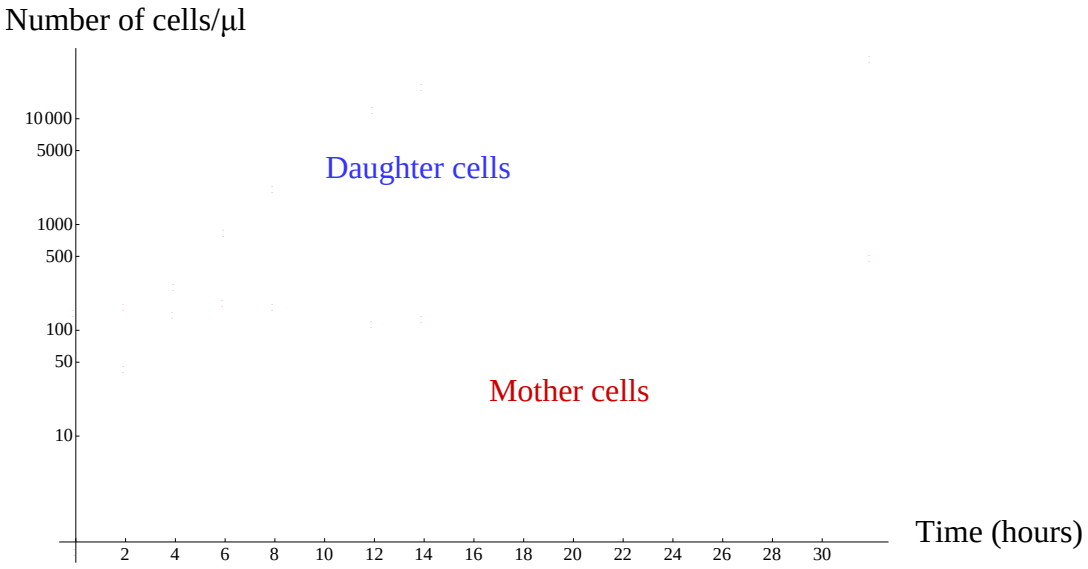

Figure 8. The number of mother cells (red) stays constant whereas the amount of daughters (blue) increases

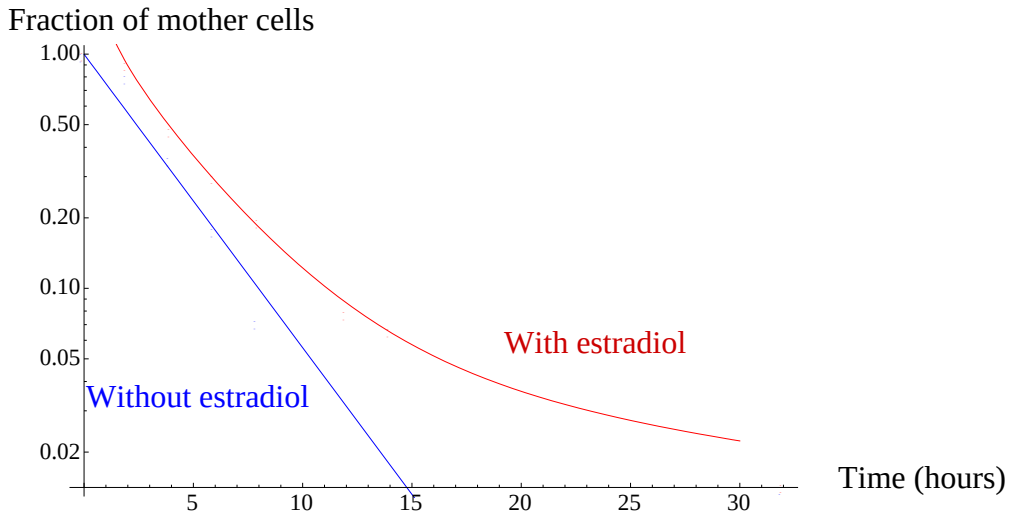

Figure 9. The fraction of mother cells decreases exponentially in a culture without estradiol (blue). It decreases a lot slower (but not yet linear) in a culture with estradiol

Then we thought maybe the MEP would work better with a higher concentration of estradiol. Therefore we made growth curves with different concentrations of estradiol. We then found that increasing the estradiol concentration did not improve the MEP (Figure 10).

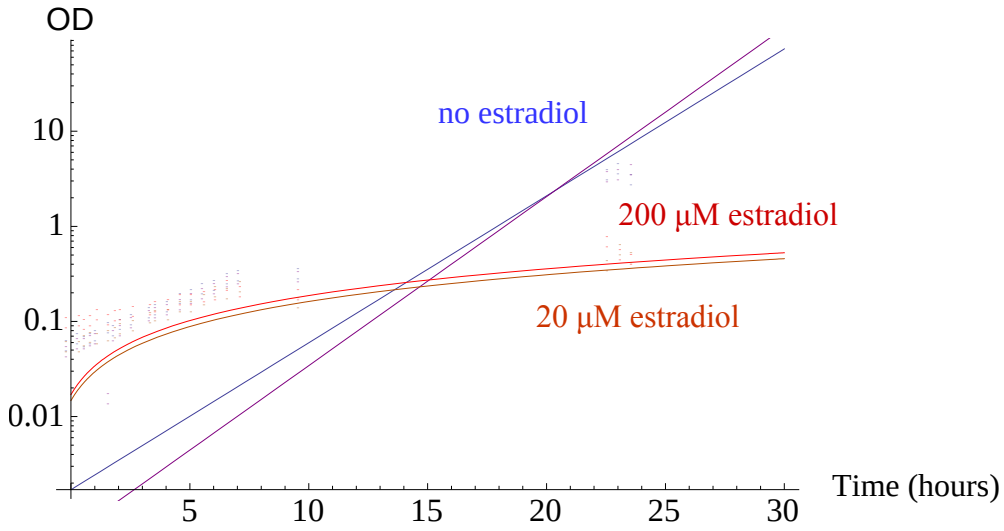

Figure 10. The growth of yeast with high and low concentrations of estradiol

Next we wanted to know what happened to the glucose concentration over time. Therefore we measured the glucose concentrations with a glucose (HK) estimation kit. We found that the glucose concentration decreased (as expected, since the cells are consuming glucose). Furthermore we found that the glucose consumption per cell decreased (since less glucose is available). This decrease was faster in the culture with estradiol.

Glucose consumption

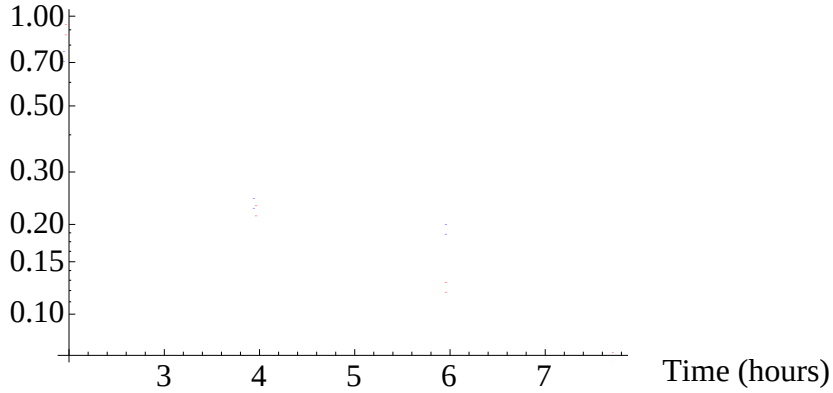

Figure 12. The glucose consumption per cell per hour in the culture with (red) and without (blue) estradiol.

Conclusions and outlook

From this research we can conclude that the mother enrichment program is working in the 5185 strain. It is probably also working in the 5181 strain. Whether it is working in the 5179 and ADH2 strain remains unclear. Even in the 5185 strain, it is however not yet perfect. Adding more estradiol does not improve the MEP.

The mother enrichment program can be used to study ageing in yeast. Once the old cells are isolated the difference with younger cells (in for example protein concentration, metabolites, etc.) can be studied.

Acknowledgements

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References

[1] Steinkraus, K. A., Kaeberlein, M. & Kennedy, B. K. Replicative aging in yeast: the means to the end. *Annu Rev Cell Dev Biol* **24**, 29---54, doi:10.1146/annurev.cellbio.23.090506.123509 (2008).

[2] Maris, A.F. Assumpçâo, A.L.K., Bonatto, Brendel, D., Antônio, J., Henriques, J., Diauxic shift-induced stress resistance against hydroperoxides in *Saccharomyces cerevisiae* is not an adaptive stress response and does not depend on functional mitochondria. Curr Genet 39, 137-149, doi:10.1007/s002940100194 (2001)

[3] Lindstrom, D.L.&Gottschling, D.E. The mother enrichment program: a geneticsystem for facile replicative life span analysis in Saccharomyces cerevisiae. *Genetics* **183**, 413-422, 411SI-413SI, doi:genetics.109.106229 [pii] 10.1534/genetics.109.106229 (2009).

[4] Sinclair DA, Guarente L. Extrachromosomal rDNA circles--a cause of aging in yeast. Cell 1997;91:1033–1042. [PubMed: 9428525]

[5] Henderson, K. A. & Gottschling, D. E. A mother's sacrifice: what is she keeping for herself? *Curr Opin Cell Biol* 20, 723---728, doi:S0955---0674(08)00163--- 4 [pii]10.1016/j.ceb.2008.09.004 (2008).]

Attachments

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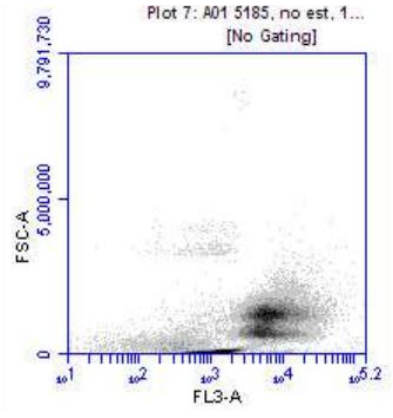

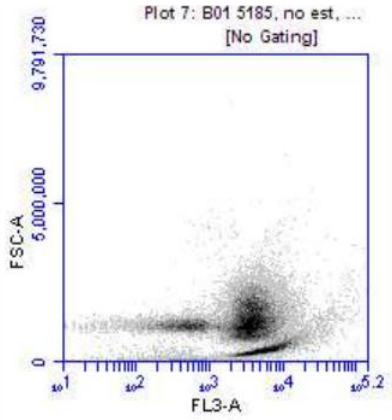

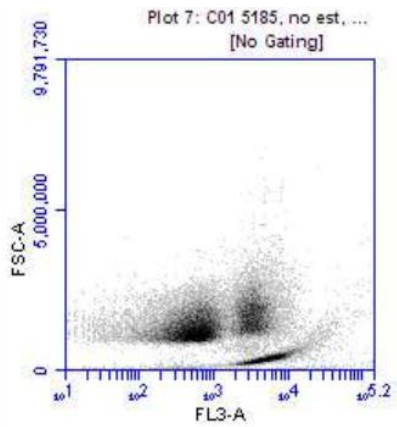

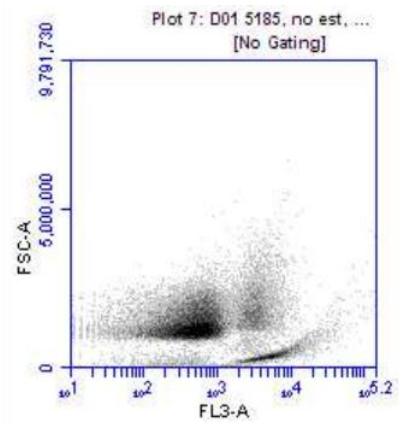

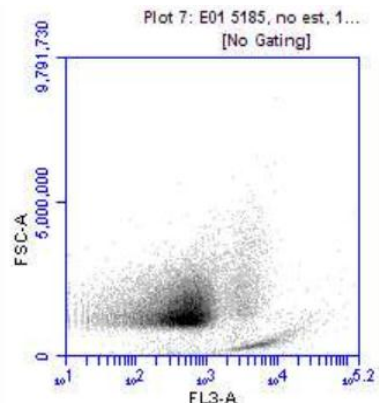

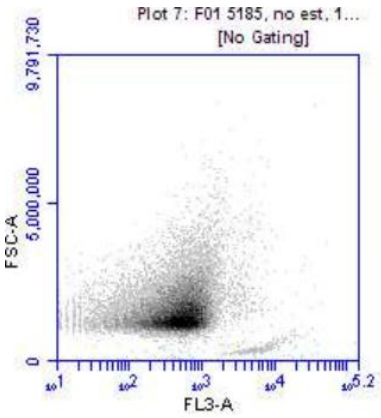

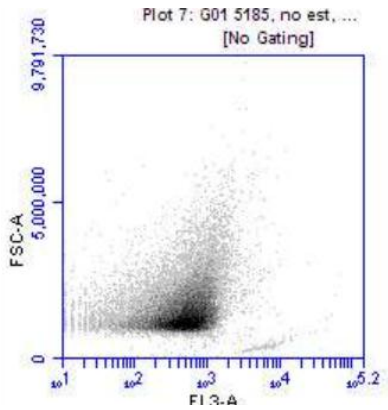

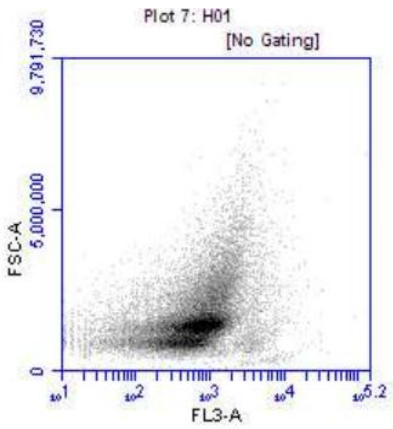

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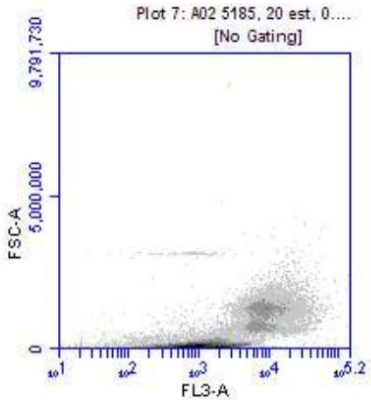
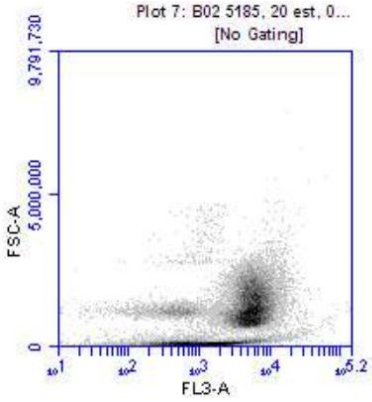
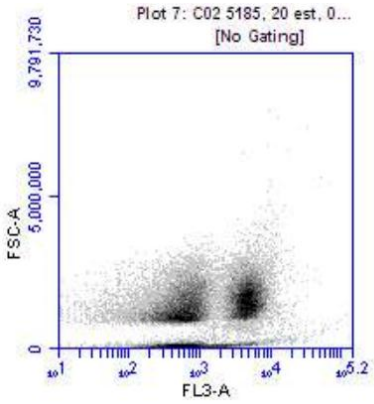
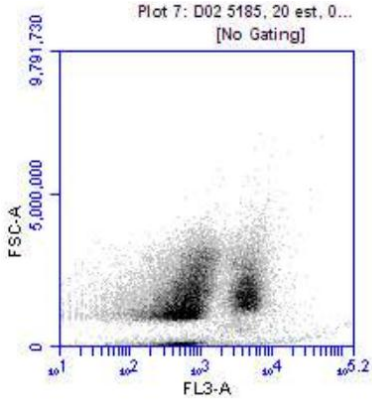
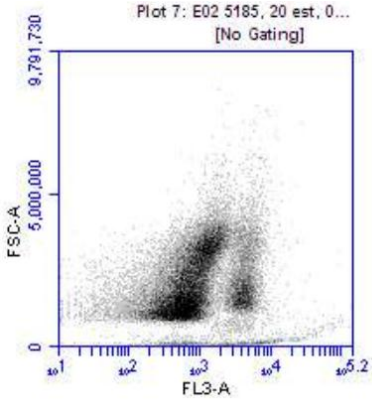
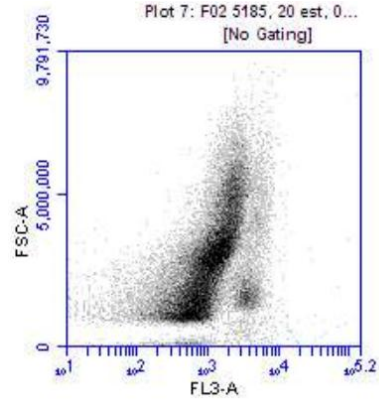


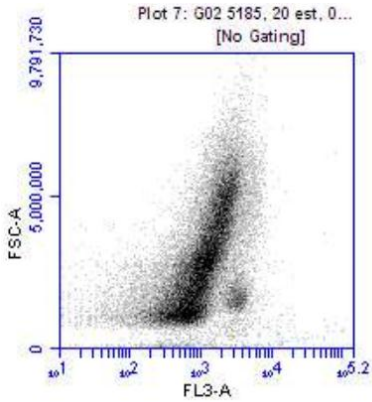


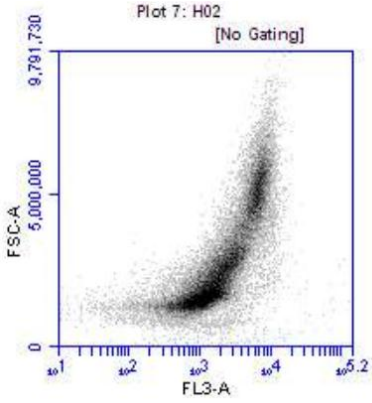




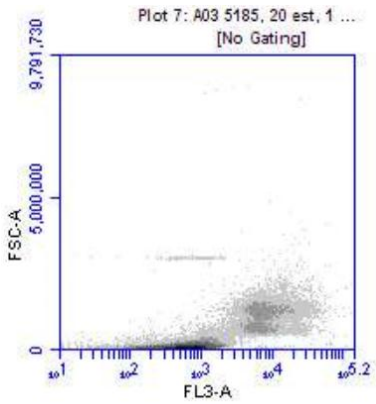


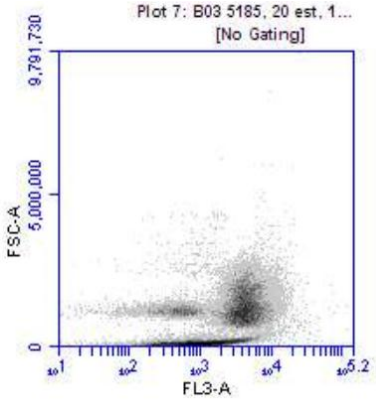


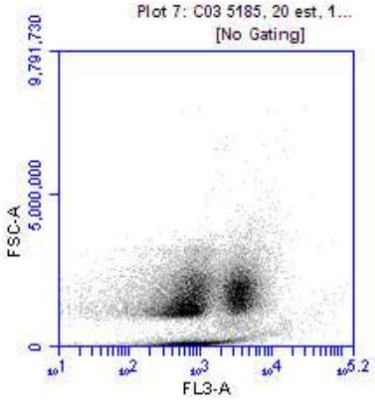


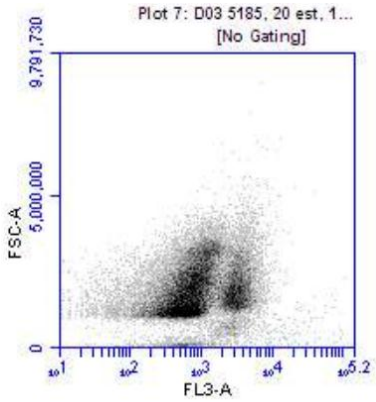


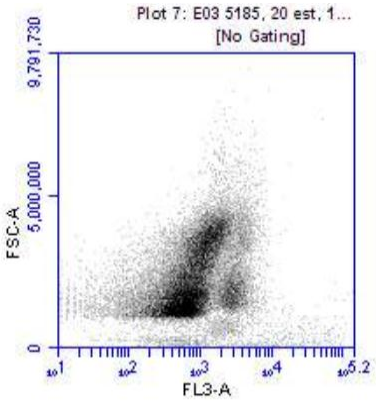
FSC-A/FL3-A 5185 with estradiol 1% glucose

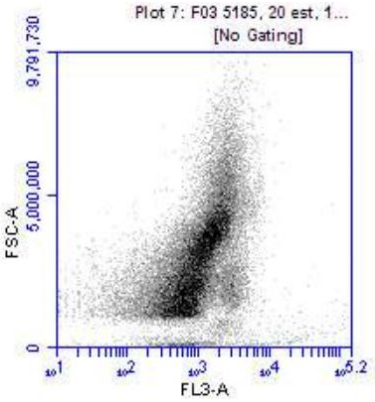


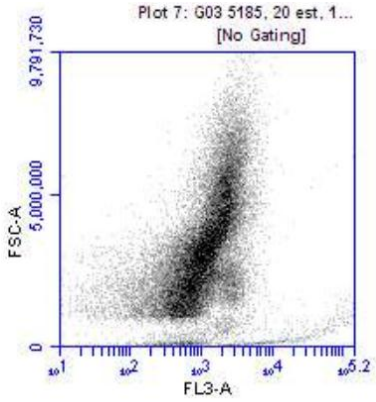


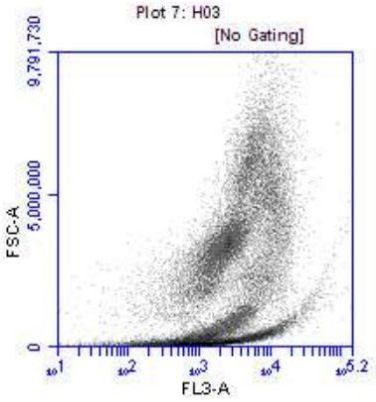




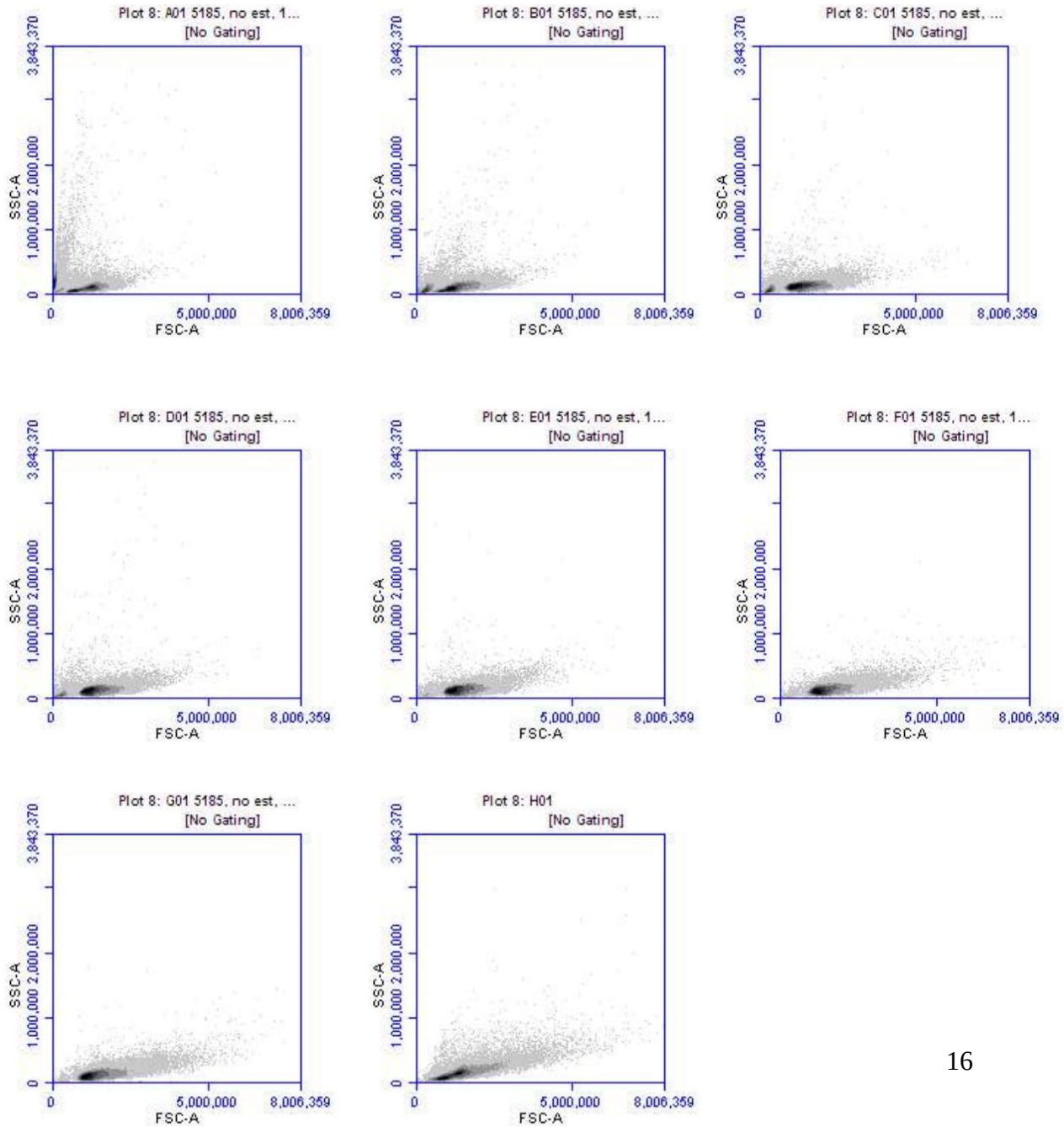




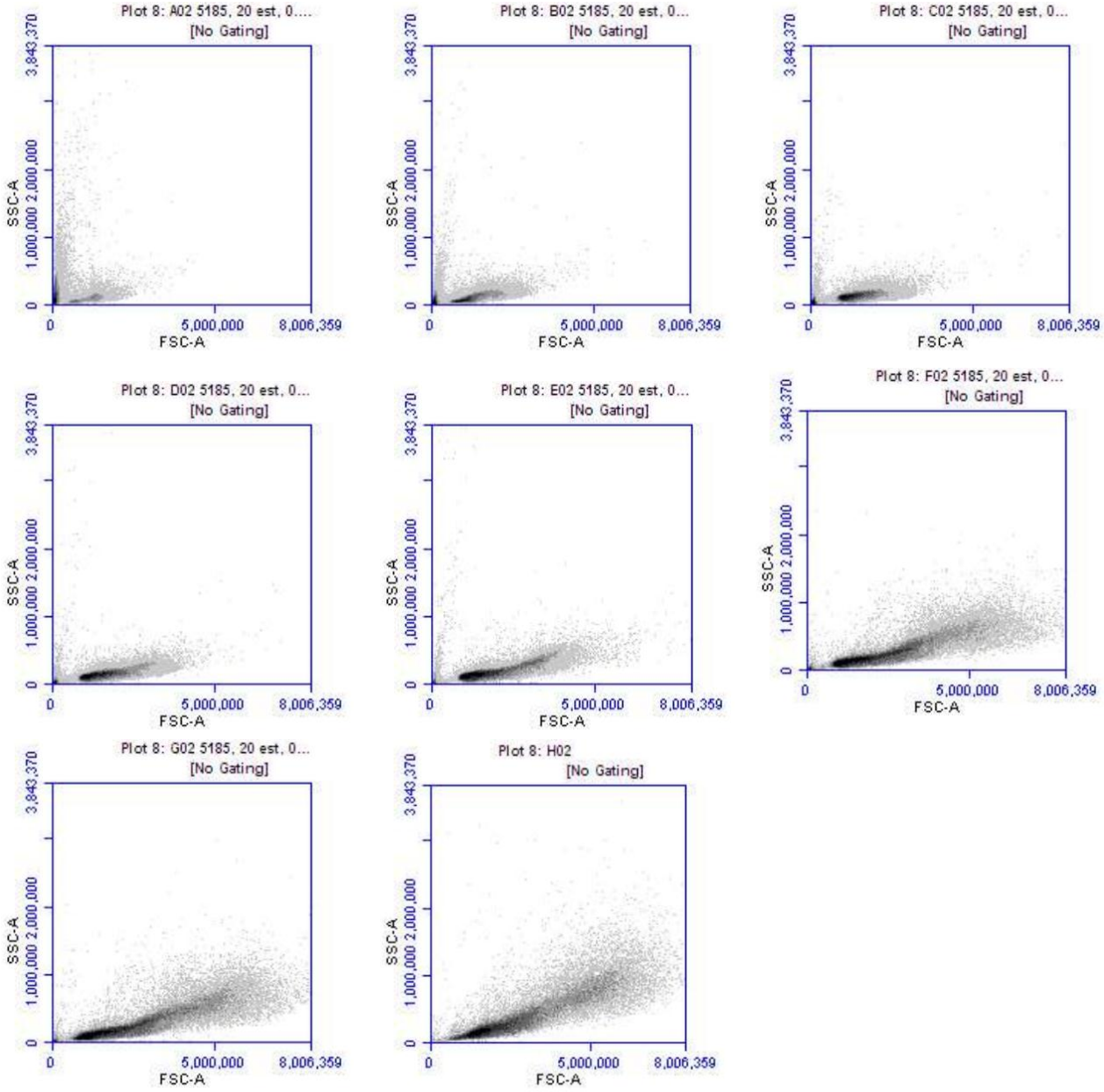




FSC-A/SSC-A 5185 without estradiol 1% glucose



FSC-A/SSC-A 5185 without estradiol 0.1% glucose



FSC-A/SSC-A 5185 with estradiol 1% glucose