Generating a Fob1-mScarletI fusion protein to image ribosomal DNA using fluorescence microscopy

Introduction:

The yeast ribosomal DNA (rDNA) locus has about 150 tandem repeats of identical sequences[1]. Each repeat contains an RFB site[2] that allows binding of a protein called Fob1 once per individual rDNA repeat. The number of rDNA repeats is linked with fitness with higher number of repeats indicative of higher fitness while lower number of repeats indicate lower fitness and ageing. Hence, estimating the number of rDNA repeats in an organism is important to estimate its fitness. Owing to the highly identical nature of rDNA repeat sequences, it is impossible to estimate the number of repeats using modern high-throughput sequencing methods. Traditionally, more laborious electrophoresis methods have been used to estimate the numbers of these repeats[3]. In the current project, we aimed to develop an in vivo Fob1-based imaging method to estimate the number of rDNA repeats in *Saccharomyces cerevisiae*. Since the Fob1 protein binds just once per rDNA repeat it is possible to estimate the number of repeats by "counting" the frequency of Fob1 binding in yeast using fluorescent microscopy.

Aims and objectives:

The aim of this project was to estimate the number of rDNA repeats using Slimfield fluorescent microscopy[4]. To achieve this, we set out to tag the Fob1 protein with a fluorescent protein mScarletI. We aimed to introduce the resulting fluorescent fusion into a suite of yeast strains carrying varying number of rDNA repeats and estimate the frequency of Fob1 binding to DNA by observing the relative fluorescence intensities of molecules bound to DNA. These intensities would give a readout of the frequency of Fob1 protein binding to rDNA.

The following were the objectives of the project:

1) Generation of Fob1-mScarletI fusion by infusion cloning: Cloning of Fob1 into an mScarlet-I plasmid and characterisation of clones
2) Introduction of positive clones into yeast and imaging the resulting strains.

Methods:

The methods used in this project were largely molecular biology related. I generated DNA fragments from yeast genomic DNA and cloned them using standard techniques such as PCR, Infusion cloning, transformation and colony screen using EcoRI digestion. I was able to generate and screen the recombinant clones myself, and also had the opportunity to image cells from the existing stocks using Single-molecule Slimfield fluorescence microscopy.

Results:

To generate the Fob1-mScarletI fusion, a pair of PCR primers with homologies to the rDNA binding protein Fob1 were designed to amplify a plasmid carrying the gene encoding mScarletI fluorescent protein. Another pair of primers were designed to amplify Fob1 such that Infusion cloning (Takara Biosciences) of the two PCR products generated an in-frame mScarletI-fob1 fusion on the resulting plasmid. Clones were selected by transforming the infusion

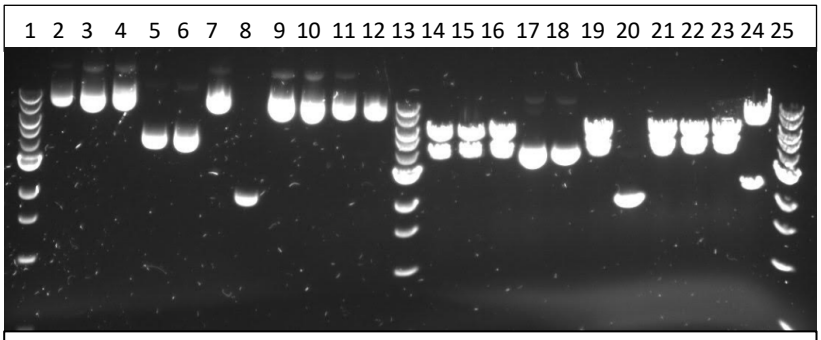

Figure 1: Restriction digestion of mScarletI-Fob1 clones to screen for positive clones. Plasmid clones were digested with EcoRI enzyme and analysed on a 0.8% agarose gel.
Legend: lanes 2-12: uncut plasmids: 14-24: plasmids digested with EcoRI; lanes 1, 13 and 25: 1Kb ladder. Lanes 1, 2, 3, 6, 8, 9 and 10 show bands corresponding to the expected 5.3 and 3.8 Kb, indicating that these are the correct clones.

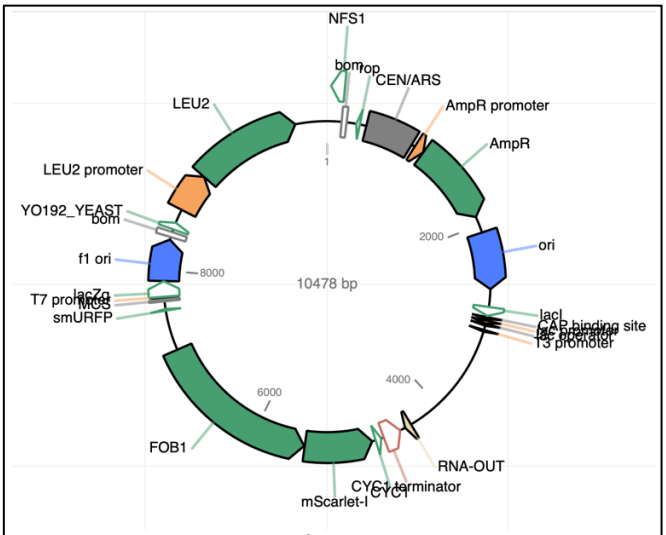

Figure 2: Plasmid map of a positive clone carrying the Fob1-mScarletI in-frame fusion.

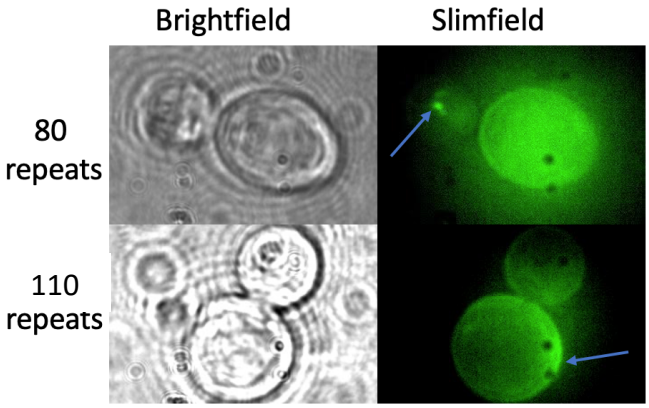

Figure 1: Images of Fob-GFP carrying yeast cells with 80 or 110 repeats of rDNA imaged under slimfield microscopy. Arrows indicate Fob1-GFP foci.

cloning mix into commercially available competent *E. coli* cells and screening by restriction digestion with EcoRI enzyme (figure 1). Positive clones were then confirmed by sequencing.

Four of the positive clones were sequenced to confirm that the fusions in-frame. All clones carried the expected in-frame Fob1-mScarletI fusion. Representative results from one clone sequence are illustrated in Figure 2.

Conclusions:

I successfully generated Fob1-mScarlet-I clones. The resulting clones can be used to quantify the number of rDNA repeats using Slimfield microscopy. I also successfully visualised the Fob1-mGFP clones using the Slimfield microscope.

Future directions:

Due to the short time frame of the project, I was unable to transform the verified Fob1-mScarletI clone into yeast test strains. However, I was able to generate preliminary images from the existing laboratory stocks using Slimfield microscopy. Yeast cells carrying an existing Fob1-mGFP fusion were imaged under a Slimfield microscope using 561nm laser at 5 ms exposure time to estimate the number of rDNA repeats that are bound by Fob1-GFP in yeast strains. I was able to visualise Fob1-mGFP foci in these cells (figure 3).

The next step of the project would be to introduce the Fob1-mScarlet-I plasmid into yeast laboratory strains carrying varying numbers of rDNA repeats and estimating rDNA copy number using Slimfield fluorescence microscopy. This would inform us of the feasibility of this approach to estimate rDNA copy number.

Value of studentship:

Student: Overall, it was an extremely enjoyable and informative experience. I have gained many technical lab skills including handling and measuring nucleic acid products as well as transforming them to host vectors. I encountered equipment such as microscopes that I have not used before. Over the course of the studentship I have become much more confident in the lab. I have been able to get to experience what life could be like after graduating. The studentship has prepared me to start my final year project at university.

Supervisor: I really enjoyed supervising Zara over the summer. She is enthusiastic, has a keen eye for detail and picked up techniques quickly. Despite being on a short duration of only six weeks she made significant progress and successfully cloned Fob1-mScarlet-I and characterised the resulting clones. Thanks to Zara's efforts, I now have a construct that I can use to generate preliminary data for my fellowship applications.

References:

1. Proc. Natl Acad. Sci. USA (1979) 76, 410 –414.
2. Genes to Cells (1996) 1, 465–474
3. Science (2010) 327, 693-696.
4. Integrative Biology (2009) 1(10), 602-12.