Exploiting SCRaMbLE to increase fatty acid synthesis in yeast

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A thesis submitted in fulfilment for the degree of Master of Research

14 October 2018

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Abstract

Fatty acids (FAs) and lipids produced in yeast could provide an alternative source for consumable oils in the future. In this work, four recombinant yeast strains were developed using The SCRaMbLEing technology (Synthetic Chromosome Recombination and Modification by Lox-P Mediated Evolution) developed in the global Yeast 2.0 project aiming to build a completely synthetic *Saccharomyces cerevisiae*. The genes: *ACC*1 (acetyl-CoA carboxylase 1), *ACS*1 (acetyl-CoA synthetase), *FAS*1 (fatty acid synthase 1) and *FAS*2 (fatty acid synthase 2) enclosed by loxP sites were overexpressed in a semi-synthetic yeast strain equipped with a synthetic chromosome 14 containing about 260 Lox-P sites to enable SCRaMbLE. The lipid stain Nile red stain for neutral lipids was successfully used in conjunction with flow cytometry to develop a high through put screening protocol for yeast strains with increased FA content. The FA profiles of the SCRaMbLEd strains Syn-sXIV, Syn-sACC1, and Syns-FAS2 were then analysed by gas chromatography mass spectrometry and an increase in C16:0, C16:1, C18:0 and C18:1 FA content was detected in strains expressing *ACC*1, and *FAS*2.

Declaration

The work presented in this thesis was carried out in the fulfilment of the degree of Master of Research at Macquarie University. This material has not been submitted for assessment or in the attainment of qualifications to any other university or institution.

This thesis is my own original work, undertaken by me between January 2018 and October 2018. It contains no material previously written or published. Contributions made by other authors have been cited with references to the literature. Assistance and instructions made by others have been acknowledged.

This research was undertaken with approval under Institutional Biosafety Committee (IBC) with the Yeast 2.0 5201401059 licence.

Thomas Robert Collier

October 2018

Acknowledgments

First, I would like to express my deepest appreciation to my supervisor Prof. Helena Nevalainen not only for taking on the challenge of this masters in her last year in academia, but also for guiding me through the process, for celebrating my wins and encouraging me during me losses. You are truly an inspiration and without your help I would not have completed this thesis.

I would like also especially thank Dr. Hugh Goold, who has train me to be a better scientist, through keeping me on track, his wealth of knowledge and endless advice and encouragement. Additionally, for his generous and selfless involvement in the project and its design and implementation for which this thesis would have never occurred without his valued input. I would also like to thank Dr. Anglea Sun for her continued input, support and help with writing this thesis.

I would like to thank Thomas Williams for allowing me to use the synthetic yeast strain and various plasmids. I would like to thank Ian Paulsen for allowing me to use his laboratory and equipment. I would like to thank all my colleagues and lab members, Paul Jaschke and Dr. Liisa Kautto for their training and management of the laboratory. To Alexander Carpenter, Andras Hutvagner, Briardo Llorente, Dominic Logel, Elizabeth Wightman, Geraldine Sullivan, Heinrich Kroukamp, John Timmins, Kai Peng, Monica Espinosa Gomez, Niel van Wyk, Russel Vincent, Xin Xu, Roy Walker, Patrick Da Roza I thank you for your daily advice and friendship. I would like to express my appreciation to the members of the Macquarie University Flow Cytometry Facility. In particular, to Dr. Martin Ostrowski who has been a mentor and allowed me to develop my skills in the lab and Patrick Da Roza who helped me throughout the project.

Most importantly, I am grateful for my beautiful, amazing, kind, passionate partner Alia who's continued support, love and encouragement has enabled me to never give up and realise that this is just a masters and she is my life. Secondly, I would like to express my thanks to Alia again for her passion and love for the orangutan, as without her the endeavour to find an alternative source for palm oil would not have begun.

Sincerely,
Thomas Collier

Abbreviations

ER	Endoplasmic reticulum
DAG	Diacylglycerol
FA	Fatty Acids
FSC-A	Forward scatter – Area. Used to detect cell size.
FCM	Flow cytometry
FACS	Fluorescence activated cell sorting
LB	Lipid Body
PBS	Phosphate buffered saline
SSC-A	Side scatter – Area. FCM parameter used to detect cell granularity.
SCRaMbLE	Synthetic Chromosome Recombination and Modification by LoxP-mediated Evolution
TAG	Triacylglycerides
TAGs-A	Emission band pass used to detect chlorophyll fluorescence. Ex 488 nm, Em 580/30 nm.
TAGs	Emission band pass used to detect chlorophyll fluorescence. Ex 488 nm, Em 584/42 nm.

Chapter 1 Introduction

Palm oil is a versatile, inexpensive, oxidative stable commercial oil widely used for cooking, and present in up to 50% of all grocery store products from air fresheners to shampoos [1]. This is due to the high concentration of saturated fats, such as palmitic acid (C16:0) from which the oil takes its name, and oleic acid (C18:1) [2]. However, the production of palm oil comes at a cost. It is associated with mass deforestation, habitat loss, and species endangerment and extinction. Currently, 90% of global palm oil production comes from the once rainforested areas of Indonesia, Thailand and Malaysia. If current trends continue, most of these rainforests will be lost over the next few decades [3]. The trend of mass deforestation has already led to species endangerment and loss; for example, the Sumatran orangutan was recently listed as critically endangered and predicted to be extinct within the next decade. As an additional side effect of habitat loss, palm oil plantations result in substantial greenhouse gas emissions, as most plantations are built on peatlands that act as carbon sinks. When the peatland is cleared for plantations, the CO_2 is released, contributing to global warming [4]. The aim of this project is to remedy this problem by developing an environmentally friendly alternative solution for palm oil production.

1.1 Choosing a molecular biology solution

One alternative to palm oil plantations would be to develop recombinant palm plants, capable of producing a significantly higher yield in order to use existing plantations while reducing the spread of deforestation. Additionally, this would use the existing infrastructure and reduce the economic burden of developing a commercial replacement. Another solution would be to combine the photoautotrophic abilities of plants and the production of oils like oleic acid within photosynthetic microbe-like algae taking up minimal land area and allowing the forests to regrow. Unfortunately, both plant and algal lipid biochemistry are not yet entirely understood, therefore exploiting their natural abilities would first require defining several pathways and genes involved in fatty acid synthesis.

Alternatively, yeasts are relatively well understood at the molecular level, with some yeast species classified as naturally oleaginous, meaning oil producing. These species include *Candida 107, Cryptococcus curvatus, Lipomyces lipofer, Rhodotorula glutinis, Rhodosporidium toruloides, Trichosporon pullulans* and *Yarrowia lipolytica* [5]. The oleaginous yeast *Yarrowia lipolytica* holds the most promise, as it can store up to 73% of its

cell weight in triacylglycerides (TAGs), a neutral lipid compound often made up of long chain fatty acids like palmitic and oleic acid [6]. Therefore, *Yarrowia lipolytica* would be an ideal platform for oil production mostly in the form of TAGs. However, the lack of well-established molecular tools is a limiting factor in engineering *Yarrowia lipolytica*. Regrettably, with the molecular tools at present to engineer *Yarrowia lipolytica* would take too long to save animal species like the wild Sumatran orangutan. When compared with plants, algae and oleaginous yeasts, the model eukaryotic organism *Saccharomyces cerevisiae* (the baker's yeast) seems to be the most practical candidate for genetic engineering [7], with the goal to provide a synthetic alternative for oil production.

1.2 Choosing *S. cerevisiae* as a fatty acid and lipid synthesis platform

Although *S. cerevisiae* is not the most prolific lipid producer, it is one of the most thoroughly understood eukaryotic organisms at the molecular level. *S. cerevisiae* also has a growth advantage as a single-celled organism with a short generation time compared to most eukaryotic organisms, which means that multiple variant strains can be generated in a short time, thus offering a shorter turnaround time for production. Having *S. cerevisiae* transformants in their haploid states would also help maintain strain stability and genetic uniformity [8]. Additionally, the ability to transform *S. cerevisiae* with new genes, or to perform gene knockouts through homologous recombination, can be performed routinely [9]. *S. cerevisiae* shares many cellular complexities of plants and animals without as much non-coding DNA to interfere with genetic manipulations and downstream molecular biology processes. Additionally, the *S. cerevisiae* genome was the first eukaryotic genome to be completely sequenced and made available within the public domain in the *Saccharomyces* Genome Database (SGD) [10]. With all of its positive attributes, *S. cerevisiae* has become a popular host strain for molecular biology and recently also a target platform for the development of lipid production.

1.3 Genetic manipulation of *S. cerevisiae* for fatty acids and lipids

S. cerevisiae has been a significant focus for the production of biofuels-based products over the past few decades [11]. Shown below in Table 1 are some of the ways the organism has been metabolically engineered to improve FA-derived products.

Table 1. Genetic manipulation of *S. cerevisiae* to enhance lipid production.

Target	Genetic Manipulation	Strain	Titre (product)	Reference
FAs	Overexpression of *TesA*, *ACC1*, *FAS1* and *FAS2*	BY4727	0.4g/l	[12]
FFA (C14.0 C16.0, C18.0)	Overexpression of *Mus musculus ACL* Deletion of *IDH1* and *IDH2*	BY4741	0.13g/l	[13]
Extracellular FFA (C16, C18)	*ΔFAA1, ΔFAA4* and *ΔFAT1* in a sextuple mutant. Overexpressed *DGA1* or *ARE1* with *TGL1, 3 or 5*	BY4741	1.2g/l, 2.2g/l	[14]
FFA (C16, C16), Phospholipid.	*FAA1/4, POX1, DGA1, LRO1, ARE1/2, PAH1, LPP1, DPP1 + 'tesA*. *FAA1/4, POX1, DGA1, LRO1, ARE1/2, PAH1, LPP1, DPP1 + PLB1/2*	CEN.PK 113-5D	130g/l DCW*, 620g/l DCW*	[15]
FFA and FAEE	Prevented TAG and SE formation and Beta oxidation FFAs, FAEE	JV03, JV01	1.5% g DCW*, 0.172/l	[16]
FFA	*ΔPOX1, ΔFAA1/4, ΔHFD1, 'TesA, Acetyl-Coa, RtFAS, TEF1p-ACC1*	YJZ47	10.4/l	[17]
FAEE (C16.0, C18.0)	Overexpression of *AbWS, ACC1, FAS1/2, ΔPOX1*.	BY4742	0.005g/l	[12]
TAGs	Overexpression of *ACC1, PAH1, DGA1 and ΔTGL3/4/5, ΔARE1 ΔPXA1, ΔGUT2, ΔPOX1*.	RF11	0.254m/l DCW*	[18]

Note: FFA (Free Fatty Acids), FAEE (Fatty Acid ethyl ester), TAG (Triacylglycerides). For the full gene names refer to Fig. 2. *DCW = Dry cell weight.

1.4 Yeast fatty acids and lipid biochemistry

1.4.1 Fatty acids

Fatty acids (FAs) are single carbon chain molecules that are incorporated in cell membranes, involved in signalling functions, make up the building blocks for lipids, and serve as energy storage [19]. Fatty acid elongation is thought to occur in the endoplasmic reticulum (ER) primarily but can also takes place in the mitochondrion. When the yeast grows on a rich carbon source, FAs are stored within lipid bodies as TAGs (Fig. 1-3) [20]. During cellular starvation, the cell catabolises the TAGs binding the three fatty acids to the glycerol molecules, and utilises them as a source of carbon for cellular processes [21].

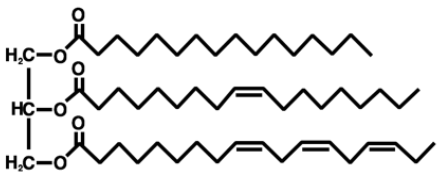

Fig. 1. *A Triacylglyceride molecule, made of a glycerol backbone (left) attached to three different fatty acids, palmitic acid, oleic acid and alfa-linolenic acid (right).*

1.4.2 The yeast fatty acid synthesis pathway, biosynthesis and regulation

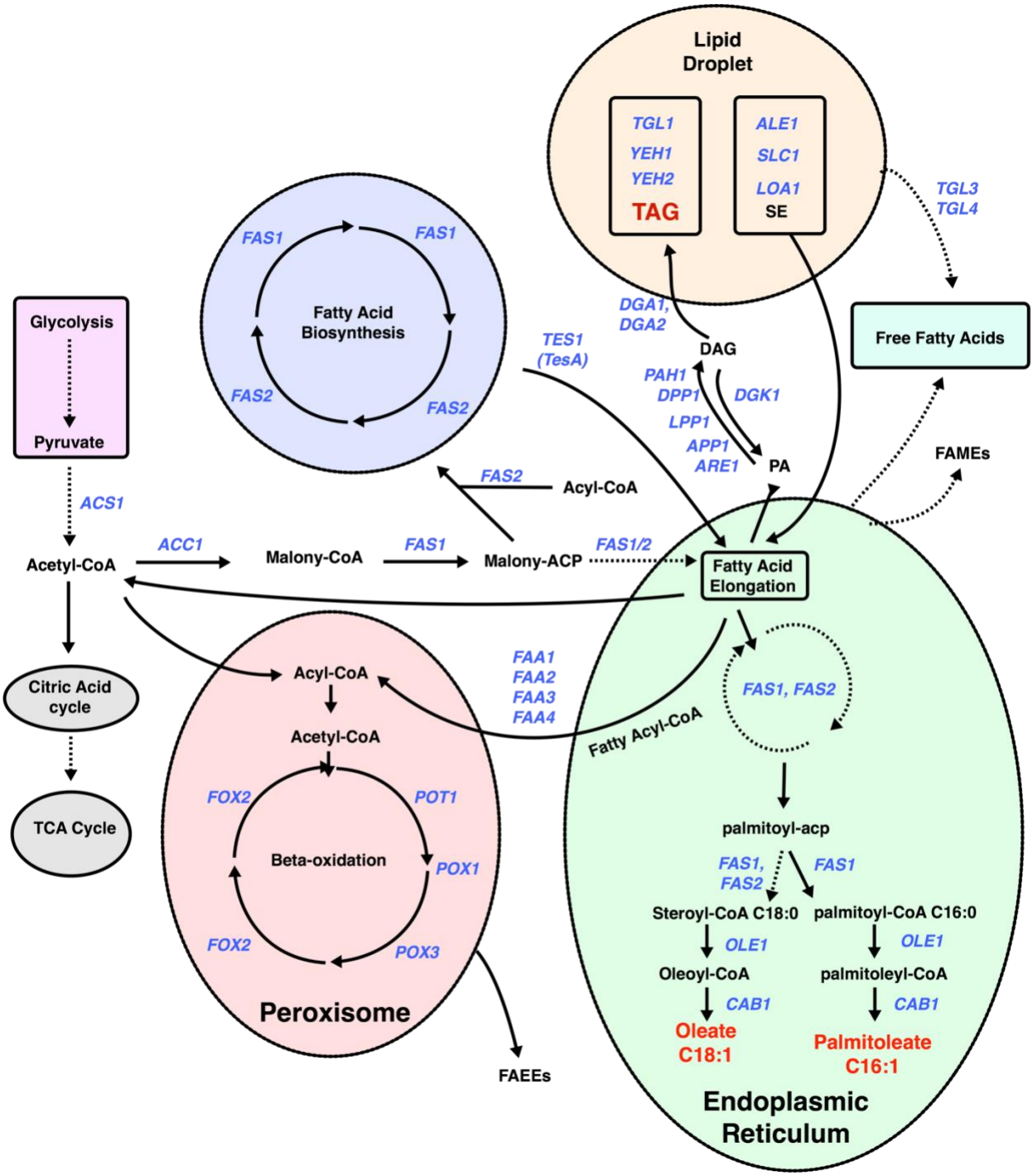

Fig. 2. *A general overview of the fatty acid synthesis pathway in yeast S. cerevisiae. Solid lines indicate a direct interaction between two genes or a gene and a product, while dashed indicate that there are several or more steps between the two genes or interactions. The genes shown in blue text represent S. cerevisiae genes. Shown in red are the desired products of interest. The genes are listed in alphabetical order: Acetyl-CoA carboxylase (ACC1) [22], Acetyl-coA synthetase (ACS1) [23], Acyltransferase (ALE1) [24], Acyl-CoA: sterol acyltransferase (ARE1) [25], Actin patch protein (APP1) [26], Pantothenate kinase (CAB1) [27], Diacylglycerol acyltransferase 1 (DGA1) [28], Diacylglycerol acyltransferase 1 (DGA2) [29], Diacylglycerol kinase (DGK1) [30], Diacylglycerol pyrophosphate (DPP1) [31], Fatty Acid Activation 1 (FAA1) [32], Fatty Acid Activation 2 (FAA2) [33], Fatty Acid Activation 3 (FAA3) [33], Fatty Acid Activation 4 (FAA4) [34], Fatty acid synthetase 1 (FAS1) [35], Fatty acid synthetase 1 (FAS2) [36], Fatty acid Oxidation (FOX2) [37], Lysophosphatidic acid (LOA1) [38], Lipid phosphate phosphatase (LPP1) [39], Fatty acid Oxidation (FOX2) [37], [40], Phosphatidic acid phosphohydrolase (PAH1) [41], Peroxisomal Oxoacyl Thiolase (POT1) [42], Fatty-acyl coenzyme A oxidase (POX1) [43], Peroxisomal Oxoacyl Thiolase (POX3) [43], SphingoLipid Compensation (SLC1) [44], ThioESterase (TES1) [40], TriGlyceride Lipase 1 (TGL1) [45], TriacylGlycerol Lipase 4 (TGL4) [46], Fatty acid Oxidation (FOX2) [37], Delta(9) fatty acid desaturase (OLE1) [47], Yeast steryl ester hydrolase 1 and 2 (YEH1 and YEH2) [45].*

In the yeast fatty acid pathway, the acetyl coenzyme A (CoA) enzyme called ACS1 catalyses the production of acetyl-CoA, the initial starting material for FA synthesis [48]. Then FAB commences with the transformation of acetyl-CoA into malonyl-CoA by the acetyl-CoA carboxylase (*ACC1*) [49]. These two precursors are then condensed by the fatty acid synthases (FASs) to fatty acids exploiting malonyl-CoA as the extender unit. Every elongation of two carbon units in FAB uses two NADPH molecules to build the fatty acids. In the cytosol of *S. cerevisiae*, FAB is catalysed by the fatty acid synthesis (FAS) system, with the functional domains made up of two subunits, encoded by *FAS1* (β-subunit) and *FAS2* (α-subunit). Since all functional domains, including the acyl carrier protein, are within the FAS complex, the entire FAB process is performed within the fatty acid elongation compartment after malonyl-CoA is loaded (Fig 2.) [50]. To recreate this process in yeast the genes *ACC1, ACS1, FAS1* and *FAS2* were chosen.

S. cerevisiae has been shown to become slightly oleaginous by uncoupling the regulatory genes △FAA1 and △FAA4 while overexpressing Acyl-CoA thioesterase (Acot5s) and Diacyl-glycerol acyltransferase (Dga1p), converting it into an oleaginous yeast [51]. In a similar feedback mechanism, high concentrations of acyl-CoA have been shown to repress the synthesis of lipids by interrupting the transcription of *ACC1, FAS1* and *OLE1* [51-53]. Additionally, oleic acid has a unique negative feedback inhibition of *OLE1* by blocking the transcription factor, and blocking the *OLE1* promoter by binding to an oleate response element (ORE). This relationship suggests *OLE1* could be exploited as an oleic acid reporter and biosensor, which could be used if incorporated into a synthetic biosensor to preliminarily diagnose strains without the need for FA extraction methods [54, 55]. All of these findings suggest the fatty acid biosynthesis in yeast is heavily regulated.

In yeast, the FA synthesis is highly regulated, and nitrogen starvation and the cell cycle have been shown to repress genes *ELO1, ELO2/FEN1* and *ELO3/SUR4* and FA chain elongation [56]. Therefore, several regulatory processes must be overcome to redirect the full utility of the FA synthesis pathway. Additionally, neutral lipids such as TAGs could be overproduced by uncoupling several regulatory mechanisms within the FA synthesis pathway [57]. This would be a practical goal as FAs and free fatty acids (FFAs) will be stored as TAGs, once the yeast cell cycle enters stationary phase [20]. Thus by uncoupling key regulatory mechanisms and by directing the carbon flux towards TAG synthesis, a greater accumulation of FAs in the form of TAGs could be achieved.

1.5 The lipid body – functions, structure and regulation

The lipid bodies (LB) are subcellular vacuoles for the storage of neutral lipids. In *S. cerevisiae*, non-polar lipids are a reservoir of energy and the building blocks for membranes. Lipid bodies are composed of a neutral lipid core surrounded by a phospholipid monolayer [58]. The LB's primary function is to encapsulate and store neutral or non-polar lipids such as TAGs and sterols or sterol esters (SE), thus protecting the cell from lipotoxicity (Fig. 3) [59].

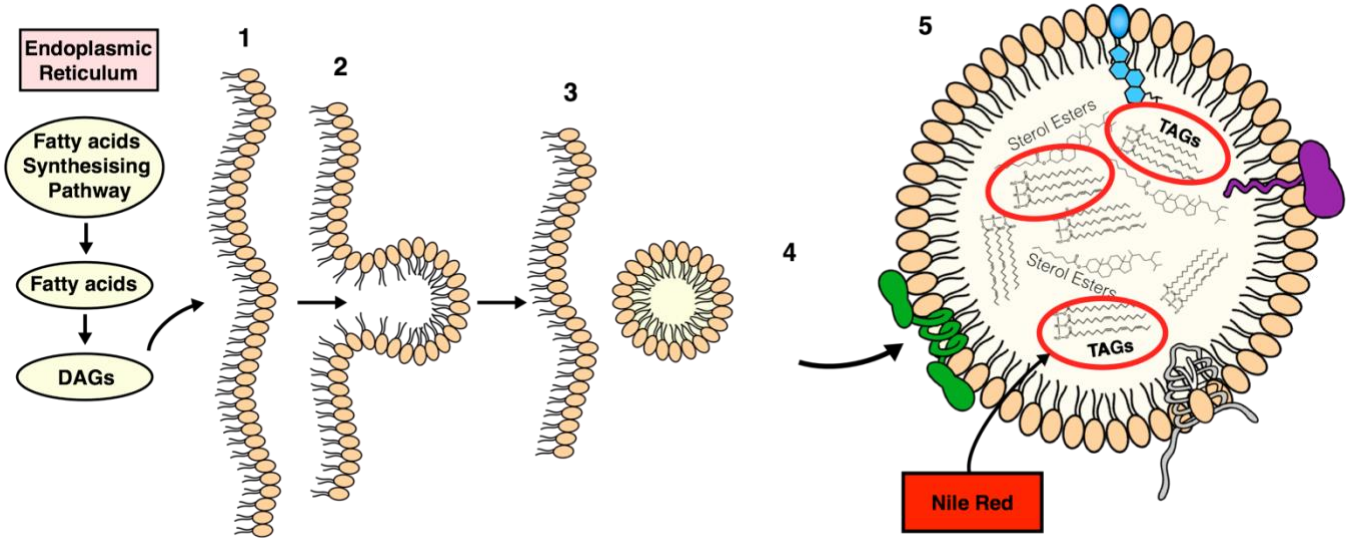

Fig. 3. *Formation of the lipid body from the ER. The process begins (1) with membrane phospholipids lipids of the ER invaginating (2) pinching off (3) to form the lipid droplet (4). The LB consists of polar phospholipids surrounding a neutral lipid core made of TAGs and SEs (5). Yeast LB are thought to have homologue proteins of perilipins or oleosines found in plant and animal LB [60]. As the lipid body forms fatty acids are packaged into diacylglycerides and then triacylglycerides. The neutral lipid stain Nile red can bind to TAG molecules identifying them within the cell [59].*

As yeast enters the stationary phase, FFA are converted into TAGs and stored within lipid bodies (Fig.1 and Fig. 2). The lipid stain Nile red selectively binds to neutral (TAGs) and polar lipids (phospholipids), therefore with Nile red staining, strains with high FA synthesis should result in greater TAGs stores which can be visualised and possibly quantified.

TAGs are generated in the subdomains of the endoplasmic reticulum from diacylglyceride (DAG) precursor molecules and transported into the growing LB (Fig. 3) [59]. The outer structure of the LB is a polar lipid monolayer of primarily cytoplasmic lipids stabilised by proteins, creating a discrete but non-transient micelle-like structure (Fig. 3) [61, 62]. The proteins attached or embedded within the outer layer change depending on cell types and complexity of different organisms. For example, prominent proteins of mammalian LB's are perilipin, adipophilin and others [63]. While the most prominent proteins of plant oil droplets in seeds are the oleosins, which cover the surface of the droplet and prevent

coalescence and LDAP are the key oil body protein in mesocarp oil bodies (palm oil) [64], in yeast LB homologues of perilipins or oleosines have not been detected [65]. However, some proteins involved in lipid metabolism were found to be characteristic for this yeast organelle [60]. Several proteins of the yeast LB can be assigned to functional groups such as phosphatidic acid biosynthesis, fatty acid activation, TAG and SE metabolism and sterol synthesis. However, the mechanism by which proteins are associated with and targeted to the LB surface in yeast has not been well documented. One favoured model suggests that TAG and SE are synthesising enzymes from significant amounts of non-polar lipids between the two leaflets of the ER, which finally results in budding newly formed LB's [66, 67]. Alternative mechanisms of LB biogenesis are also possible [68], but all sources suggest that the LB originates from the ER.

The internal composition of LB varies from the carbon source used for cell cultivation [69]. A way to make "obese" yeast cells is to grow them on oleate. With oleate growth conditions, peroxisome proliferation is induced; as it is the single subcellular fraction of *S. cerevisiae* where β-oxidation of fatty acids occurs [70]. Yeast cells that were grown on oleate also accumulate large amounts of TAGs with oleic acid as a major constituent resulting in formation of large LBs [71, 72].

So far, research into producing FAs and lipids in yeast has heavily focused on biofuel production to replace fossil fuels. Surprisingly, in yeast little or no efforts were dedicated to developing alternative consumable oils for cooking or other products. In this work, we aim to provide a proof of concept which can be used to generate alternative, economically viable, consumable oil products reliably produced in yeast.

1.6 Synthetic biology as a tool to build an oleaginous *S. cerevisiae*

Synthetic biology is a branch of genetic engineering designed to test biological systems. Synthetic biology began within bacterial systems and has now advanced to eukaryotes, including plants [73]. As synthetic biology advances for yeast (*S. cerevisiae*) and mammalian cells, gene switches and designer cells for predictable metabolic and therapeutic functions were generated, including the production of chimeric antigen receptor-modified T cells in non-immune cells [74] and RNA, and cell-based vaccines [75]. Synthetic biology approaches have shown to be tractable and useful, especially when systems are sufficiently understood, and ample research funding is available.

1.6.1 The *Saccharomyces cerevisiae* version 2.0 project

The international Synthetic Yeast Genome Project (Sc2.0 or *Saccharomyces cerevisiae version 2.0*) aims to build an artificially designed, customisable and entirely synthetic *S. cerevisiae* genome that is more stable than the wild-type. In the synthetic genome, all transposons, repetitive elements and many introns are removed, all UAG stop codons are replaced with UAA, and transfer RNA genes are moved to a novel neochromosome. The genome will have an 8% reduction in size while including 1.1 synthetic megabases of DNA encoding about 4,000 loxP sites to allow genomic recombination [76]. A synthetic genome is easily customisable, enabling quicker experiments on a genomic scale. As of March 2017, six of the 16 chromosomes have been synthesised and tested, with no significant fitness defects found [77]. Two of the yeast chromosomes, 14 and 16, are currently being built at Macquarie University, allowing for early access to a partially synthetic strain.

One critical design in the Sc2.0 are the integrated loxP sites in the synthetic chromosomes. These sites facilitate the recombination of genetic material to create large amounts of genetic diversity. The loxP sites are recognised by a Cre recombinase enabling SCRaMbLE—Synthetic Chromosome Rearrangement and Modification by LoxP-mediated Evolution—when the Cre recombinase is induced with estradiol.

1.6.2 SCRaMbLE

SCRaMbLE is an inducible evolution system that generates significant genetic diversity by randomly arranging genetic material at the pre-determined loxP recombinase recognition sites. The loxP sites are 43 base pairs, long palindromic regions flanking carefully selected genes in the genome and introduced in the vector DNA, which allows the Cre recombinase to accurately recombine DNA by either deleting, duplicating, inverting or translocating genes in equal proportions [76]. SCRaMbLE has generated yeast strains with improved xylose utilisation and penicillin or violacein biosynthesis, establishing SCRaMbLE's ability to improve diverse and unrelated pathways [78]. However, SCRaMbLEing can result in millions of unique deviations and permutations, and potentially highly desirable recombination events can be selected against if they impose a severe growth defect or could merely kill the cell, especially *in vivo*. A way to overcome this is to mate the synthetic yeast with a wild-type *S. cerevisiae* or closely related species to generate diploid strains [79]. Cross mating counteracts for the ill effects and can lead to a robust industrial strain that is partially synthetic. Another more straightforward way to select against lethal

genetic combinations is to design a sensitive phenotypic high throughput screening protocol.

1.6.3 Variations on SCRaMbLE.

The SCRaMbLE technology has come a long way with the development of Multiplexed SCRaMbLE Iterative Cycling (MuSIC) which involves multiple rounds of SCRaMbLE [80]. Alternatively, the "Reporter" of SCRaMbLEd Cells using Efficient Selection" (ReSCuES) which only report before and after SCRaMbLE allowing for relative control when recovering SCRaMbLEd cells [81]. Moreover, light SCRaMbLE (L-SCRaMbLE) that employs a light-inducible promoter enables precise control over SCRaMbLE times that prevents perishing population due to prolonged SCRaMbLE durations [139]. SCRaMbLE has also been transferred from the cell to the test-tube with an *in vitro* SCRaMbLE method [82]. This method demonstrates that by isolating a pathway or set of genes away from the complexity of the genome, SCRaMbLE can be used to rapidly probe variations in a single pathway. SCRaMbLE is an example of the many potential innovations made possible by rationally designing a species from the genome level. The principles behind SCRaMbLE, including the *in vitro* prototyping of rearranged pathways, could be expanded to other eukaryotic species such as algal or plant species in the future.

1.7 Objectives of the study

The overall aim was to develop a platform to exploit the SCRaMbLE and Yeast 2.0 technologies to produce yeast transformants capable of producing increased amounts of fatty acids. Transformants containing the four selected *S. cerevisiae* genes *ACC1, ACS1, FAS1* and *FAS2* were created and used in conjunction with flow cytometry to develop a screening method for lipid productivity. The recombinant strains were then SCRaMbLEd and applied to the screening protocol to isolate desired high lipid producing strains. The steps to achieve this goal are outlined below.

1. Select and isolate genes relevant for palmitic acid and oleic acid biosynthesis.
2. Clone the *S. cerevisiae* FA synthesis genes *ACC1, ACS1, FAS1* and *FAS2* into a plasmid which can be SCRaMbLEd.

3. Develop an efficient robust screening method using the recombinant strains overexpressing the yeast FA synthesis genes. Form a gating procedure to capture high lipid producing strains.
4. Develop a fatty acid profile for non-SCRaMbLEd recombinant strains to set a baseline against which to measure the effects of SCRaMbLE.
5. Establish a relationship between the screening method based on fluorescence and the actual fatty acid content in the strains.
6. Apply SCRaMbLE to the recombinant yeast strains and validate the developed screening protocol.

Chapter 2 Materials and Methods

2.1 Commonly used media and reagents

Media recipes were obtained from the book, *Molecular Cloning*, unless otherwise stated [83]. All reagents were prepared using sterilised Milli-Q water generated by a Milli-Q Gradient system (Millipore).

Table 2. Commonly used media in this study

Name	Contents
Yeast Peptone Dextrose (YPD)	2% (w/v)* glucose, 2% peptone, 1% yeast extract and 2% agar (for plates)
Lysogeny broth (LB)	Tryptone 1% yeast extract 0.5%, NaCl in 1l Milli-Q water, pH to 7 with NaOH If making agar plates add agar 2% (20 g/l), Cool to 50-55 ^{o}C in a water bath
Synthetic Minimal Medium (SC-)(Drop out media)	2% Glucose, 2% Agar, 6.8 g/l yeast nitrogen base – no amino acids Added amino acids to suit: adenine hemisulfate (100 mg/100 ml), uracil (200 mg/100 ml), L-histidine, L-methionine (500 mg/100 ml), L-arginine, L-leucine, L-lysine, L-tryptophan (1 g/ 100 ml)

Phosphate buffer saline (PBS)	800 ml of distilled water: 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium phosphate, 0.24 g of monopotassium phosphate, pH adjusted to 7.4 with hydrochloric acid, add distilled water to a total volume of 1 litre, sterilise by autoclaving
TAE - Tris-acetic acid and ethylenediaminetetraacetic acid (EDTA).	A 50x stock was made: 242 g tris base, 750 ml deionised water, 57.1 ml glacial acetic acid, 0.5 M EDTA (pH 8.0) up to 1 L with deionised water with a pH of 8.5
Transformation solution	2.4 ml PEG 50%, 360 µl 0.1 M Lithium acetate

Note, All solutions (except TAE) were autoclaved in a 1 L flask for 20 minutes at 121° C. Once cooled to 50-55° C in a water bath, 10 ml 20% sterile glucose was added to all yeast media and 100 µg/ml of ampicillin to LB selective medium. * All % values refer to w/v.

2.2 Primers, plasmids and strains

Primers, plasmids, and strains used in this study are shown in Tables 2, 3, and 4, respectively. Strains were constructed by transforming plasmids into the appropriate yeast strain using the lithium acetate method [9] and selecting for growth on appropriate auxotrophic dropout agar plates. All *in silico* cloning, Yeast assembly, Gibson assembly and primer design were carried out using the Geneious Pro software, version 9.1.5 [84].

Table 3. Primers used in this study

Primer number/name	5' to 3' Sequence
1/Plasmid Forward	TTGATTGCTACTGGTTCTATGTTGGGTTAA
2/Plasmid Reverse	TTGTAATTAAAACTTAGATTAGATTGCTATGCT TCTTTC
3/ACC1 Forward	GAAAGAAAGCATAGCAATCTAATCTAAGTTTTAATTACA AATGAGCGAAGAAAGCTTATTCGAGTCTTCTC
4ACC1 Reverse	TTAACCCAACATAGAACCAGTAGCAATCAATTATTTCAA AGTCTTCAACAATTTTTCTTTATCATCGGTAG
5/ACS1 Forward	GAAAGAAAGCATAGCAATCTAATCTAAGTTTTAATTACA AATGTCGCCCTCTGCCGTACAATCATCAAA

6/ACS1	Reverse	TTAACCCAACATAGAACCAGTAGCAATCAATTACAACTT GACCGAATCAATTAGATGTCTAACAATG
7/FAS1	Forward	GAAAGAAAGCATAGCAATCTAATCTAAGTTTTAATTACA AATGGACGCTTACTCCACAAGACCATTAAC
8/FAS1	Reverse	TTAACCCAACATAGAACCAGTAGCAATCAATTAGGATTG TTCATACTTTTCCCAGTTGTCGATG
9/FAS2	Forward	GAAAGAAAGCATAGCAATCTAATCTAAGTTTTAATTACA AATGAAGCCGGAAGTTGAGCAAGAATTAGCT
10/FAS2	Reverse	TTAACCCAACATAGAACCAGTAGCAATCAACTATTTCTT AGTAGAAACGGCGACCGC
11/ACS1	internal Forward	GCGGCATAGCGACGGCAG
12/ACS1	internal Reverse	GTAGCAATTGCGGGCTGCAGTCTAT
13/ACC1	internal Reverse	GGGGTGGCCATGGCGACGAA
14/ACC1	internal Forward	GATGATAGGCAAGTCGCAAC
15/FAS1	internal Reverse	CACTAGTTCAGCAGGTGTGGTAGG
16/FAS1	internal Reverse	ACATAGAACCAGTAGCAATCGTG
17/FAS2	internal Forward	CTTGGACCCATTAGCCCGTGTA
18/FAS2	internal Reverse	AAGGACCGATTTCAACAACCCTTTCAGTGTT
19/Gene	Sfi Forward*	ATGGTCATAGGGCCATATAGGCCAACGACGGCCAGTGAA TTCGGATC
20/Gene	Sfi Reverse*	CAGGAAACAGGGCCTCTCTGGCCGAGCAGCTATGACCAT GATTACGCC
21/Backbone Sfi Forward*		CTGTTTCCTGTGTGAAATTGTTATCCGCTC
22/Backbone Sfi Reverse*		CTATGACCATGATTACGCCAAGCGC

*The primers “Gene Sfi Fwd/Rev” were named accordingly as when used together they will amplify the gene (insert) region of the plasmid, while the “Backbone Sfi Fwd/Rev” primers will amplify from the plasmid backbone region. Additionally, a SfiI restriction enzyme site was incorporated into the primer to enable any two of the plasmids to be amplified and joined together if needed.

Table 4. Plasmids used in the study

Name	Genotype, Plasmid	Notes	Origin
pRS416	Yeast centromeric plasmid, URA3 marker	Main plasmid backbone used for all strains. The plasmid contains a modified *TEF1* promoter and synthetic (synth25) terminator	Euroscarf [85]
pRS416-*ACC1*	TEF1-*ACC1*-synth25-pRS416	Constructed to SCRaMbLE *ACC1*	This Study
pRS416-*ACS1*	TEF1-*ACS1*-synth25-pRS416	Constructed to SCRaMbLE *ACS1*	This Study
pRS416-*FAS1*	TEF1-*FAS1*-synth25-pRS416	Constructed to SCRaMbLE *FAS1*	This Study
pRS416-*FAS2*	TEF1-*FAS2*-synth25-pRS416	Constructed to SCRaMbLE *FAS2*	This Study
pLM006_*pSCW11*	SCW11pr-CRE-EBD-Termintor	Cre Recombinase plasmid used to express the Cre recombinase	In House

Table 5. Yeast strains used in the study

Name	Genotype, Plasmid	Notes	Origin
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Haploid auxotrophic laboratory strain, mating type 'a'. Parental strain of SynXIV	Euroscarf [85]
SynXIV	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	SynXIV strain with modified chromosome XIV	Annaluru *et al.* [86]
SynXIV - *ACC1*	SynXIV-pRS416	SynXIV and a modified pRS416 plasmid with *ACC1**	This Study
SynXIV - *ACS1*	Modified pRS416	SynXIV and a modified pRS416 plasmid with *ACS1**	This Study
SynXIV - *FAS1*	Modified pRS416	SynXIV and a modified pRS416 plasmid with *FAS1**	This Study

SynXIV - *FAS2*	Modified pRS416	SynXIV and a modified pRS416 plasmid with *FAS2**	This Study

*A recombinant SynXIV strain with a modified pRS416 plasmid.

2.3 Molecular biology

2.3.1 The polymerase chain reaction (PCR)

PCR was performed according to the following protocol:

1. DNA template one µl
2. dNTPs one µl
3. 10x reaction buffer five µl
4. 5' oligonucleotide one µl
5. 3' oligonucleotide one µl
6. Water 11 µl
7. PCR was run in a final volume of 20 µl* in an Eppendorf thermocycler according to the following PCR protocols.

Table 6. PCR cycling conditions.

Type	Initial Temperature	Annealing Temperature	Elongation Temperature	Cycles	Final Elongation	Hold
Classic PCR	98 °C (30 sec)	55 °C (1 min)	72 °C (1 min/kb*)	30	72 °C (10 min)	4 °C (∞)
Touch Down PCR	98 °C (30 sec)	65 °C-50 °C -1°C each cycle	72 °C (1 min/kb*)	15	-	-
	98 °C (30 sec)	55 °C (1 min)	72 °C (1 min/kb*)	20	72 °C (10 min)	4 °C (∞)
Step up PCR	98 °C (30 sec)	50 °C-65 °C +1°C each cycle	72 °C (1 min/kb*)	15	-	-
		55 °C (1 min?)	72 °C (1 min/kb*)	20	72 °C (10 min)	4 °C (∞)

Note: Initial melting temperature and elongation time were adjusted according to the polymerase used which include Phusion® High-Fidelity, LongAmp® and Go Taq® Green Master Mix. Classic PCR was used for amplifying the gene *ACS1* and the pTEF1-ScpC-Tsynth

plasmid backbone, while Touch down PCR was used to amplify the genes *ACC1, FAS1* and *FAS2*. *Kb = kilobase pair.

2.3.2 Gel electrophoresis

Restriction digests of plasmid DNA and PCR amplicons were visualised by electrophoresis on a 1% agarose gel of 100 ml with 1x TAE buffer and eight µl SYBR safe or GelRed DNA stain. When the DNA fragments were 5 kb or above, the gel was run at 110 V or 50 to 70 min, at 110 V for 35 min when below 5 kb.

2.3.3 Gel extraction

DNA fragments were isolated by size with gel electrophoresis and gel extracted. The protocol for gel extraction of DNA amplicons was performed and followed using a Monarch Gel extraction kit (New England Biolabs, US) (Monarch®).

2.4 DNA extraction methods

2.4.1 Plasmid extraction, E. coli Mini prep

Protocols for plasmid extraction using a Monarch plasmid extraction kit were followed to extract plasmid DNA from *E. coli* cells (New England Biolabs, US) (Monarch®).

2.4.2 Crude Yeast DNA extraction

Plasmid DNA was harvested from yeast growth in a three ml overnight culture centrifuged and resuspend according to the Lithium acetate-SDS method [87]. To help with cell lysis, acid washed glass beads were added and vortexed before the DNA was isolated by centrifugation and ethanol extraction.

2.4.3 Y-PER DNA Extraction Kit

Plasmid DNA was extracted from either an overnight culture or single colonies according to the Y-PER DNA Extraction Kit instructions, Yeast DNA Extraction Kit (Thermo-Fisher Scientific, AU).

2.5 DNA assembly methods

2.5.1 Gibson assembly

Amplicons were first cloned with a 20-40 base pair overlap. The fragments were joined as follows: 10 µl of the Gibson Assembly Master Mix (2x) was used with 5-8 µl (1.5 pmol) of the

insert (gene) to 1.6-2.6 µl (0.5 pmol) backbone (plasmid) in a 3 insert:1 plasmid ratio. They were then mixed in deionised H_2O to a volume of 20 µl. The samples were then incubated in a thermocycler at 50 °C for 15 min. Following incubation, the samples were placed on ice or stored at –20 °C for subsequent transformation. The DH 5 alpha competent *E. coli* cells with 2 µl of the assembly reaction, where then transformed by the heat shock method [88].

2.5.2 Yeast assembly

Amplicons were first cloned with a 20-40 base pair overlap. DNA was transformed according to the yeast transformation protocol (section 2.6.1) with respective amplicons in a 4:1 insert (gene) to backbone (plasmid) ratio to a total of 74 µl.

2.6 Transformation methods

2.6.1 Yeast transformation

The SynXIV yeast cells were transformed by using the lithium acetate method [9] starting with a colony inoculated into a 10 ml preculture overnight. Next, the cultures were diluted to an optical density (OD) at 650 nm of 0.125 and grown for 3-4 h until an OD of 0.5 was reached. The cells were then washed with 10 ml of 0.1 M Lithium acetate and centrifuged with the supernatant removed. For each transformation, a unique transformation mix was made consisting of: 240 µl PEG 50%, 36 µl 1 M LiAc, ten µl ssDNA and up to 74 µl template DNA plus water. The cells were then resuspended in the transformation mix and incubated for 30 mins at room temperature. Following incubation, the cells were heat shocked at 42 °C for 45 min then centrifuged and resuspended in 400 µl sterile water. The cells were then diluted 1:1,000 and 1:10,000 and plated onto selective plates (100-150 µl per plate). A no DNA control was used for each transformation.

2.6.2 *E. coli* transformations

All *E. coli* transformations were performed with DH 5 alpha competent cells according to the heat shock method [88].

2.7 SCRaMbLE protocol and SCRaMbLE killing assay

2.7.1 SCRaMbLE protocol

Cells were grown overnight in selective medium at 30 °C with shaking at 200 rpm. Ten ml of YPD medium or selective media was inoculated with an overnight culture to an optical

density (OD) at 650 nm of 0.15. Once samples had reached OD 0.5, estradiol was added to a final concentration of 1 µM, and cells were shaken at 200 rpm at 30 ºC for 3 or 5 h. Cells were then washed by centrifugation at 3,000 rpm for 10 min, followed by resuspension in H_20 or phosphate buffered saline (pH 7.5) (PBS), two times. Cells were then plated in serial dilutions on a non-selective medium or stained and sorted using a BD-Influx flow cytometer (Becton Dickinson).

2.7.2 SCRaMbLE killing assay

The SCRaMbLE killing assay was performed according to the section 2.7.1. The cells were split into a group induced with estradiol or a group not induced with estradiol (controls). The cells were then incubated for either 3, 6 or 9 h, after which they were centrifuged and washed with PBS, diluted to 1:1,000 and 1:10,000 and plated onto non-selective plates. The plates were then incubated for three days at 30 ºC. The colonies were counted, and the killing rates compared.

2.8 Staining, sorting, and counting of yeast cells

2.8.1 Lipid staining with Nile red

All procedures, unless otherwise stated, were carried out at room temperature. A 60 µg/ml of Nile red in acetone working solution was used for all staining. A DMSO: PBS (1:1) solution was made in a 100 ml stock bottle. The cells were grown for 72 h at 30 ºC. Then 250 µl of each culture was transferred to a sterile 1.5 mL tube. 25 µl of DMSO: PBS (1:1) was then added with five µl (five µg/ml) Nile red in acetone and mixed. The tube was incubated in the dark for five mins. The cells were then centrifuged for two mins at 8,000 × g, and the supernatant was carefully aspirated. The cells were washed twice in one ml 1 x PBS added to the cell pellet and centrifuged. The cells were then resuspended in 500 µl sterile water or 1 x PBS [89].

2.8.2 Flow cytometry and cell sorting protocol

A Beckman Coulter CytoFLEX flow cytometer (CytoFLEX) model was used to establish the correct concentration of Nile red needed to identify TAGs, and to measure different concentrations of TAGs in the parent strain to confirm that Nile red can be used with the flow cytometer to measure TAG levels within the cell. For fluorescence-activated cell sorting (FACS), a BD Influx flow cytometer (BD Influx) was used for all cell sorting and developing the high TAG gates within all non-SCRaMbLE-d strains (SynXIV, Syn-*ACC1*, Syn-*ACS1*, Syn-

FAS1, Syn-*FAS2*). By using the same sample in both machines, it was confirmed that the differences in measurement between the two machines was minimal. Two flow cytometers were used due to varying availability. For neutral lipid (TAG) measurements, a 200 mW 488-nm laser was used for excitation with emission filters at 580/30 nm. The SynXIV populations were analysed with and without the Nile red stain to account for auto-fluorescence in parallel to the measurement of the recombinant strain populations. To accurately measure and sort different populations with flow cytometry strict gating needs to occur. A gate is a drawn line around either a desired or undesired population. A gate or several gates in series can be used to exclude or include cells depending on the user's preference. To identify the changes caused by SCRaMbLE, the TAG profiles of the strains SynXIV, Syn-*ACC1*, Syn-*ACS1*, Syn-*FAS1*, Syn-*FAS2* were gated before SCRaMbLE. These gates were then applied to the SCRaMbLEd populations to measure changes. All post-run analysis was performed using FLOWJO® with histogram overlays generated, and gates highlighted to display the results.

2.8.3 Yeast cell counting with a haemocytometer

The cells were taken from a 72 h, 10 ml culture and normalised to an OD of 1.0. Once the cells were ready for counting the "Tiefe depth profondeur" haemocytometer was washed with sterile water and a coverslip placed over the counting area. Ten µl of cells were then loaded onto the counting area between the coverslip and the haemocytometer. The cells were then counted by adding the total number of cells of five 0.2 mm^2 squares, followed by dividing by five. The average was then multiplied by 2.5 x 10^3 to give the total number of cells per ml.

2.9 Biochemical analysis of yeast strains

2.9.1 Fatty acid extraction and generation of fatty acid methyl esters (FAMEs) through a direct transmethylation method

The direct transmethylation method was adapted and modified from a Bligh and Dyer method [90]. The cells were first normalised to an optical density of 1.0 (1.50 x 10^7 cells) and counted. One ml of culture was then transferred into a glass tube with a Teflon lined lid. The cells were then centrifuged, and the supernatant decanted into waste. One ml of 5% sulphuric acid in methanol was then added with 500 µl toluene as a co-solvent and ten µl butylated hydroxyl toluene (one % stock) to preserve the samples. Five µg (one ng/µl) of heptadecanoic acid (C17:0) was added as internal standard, and the tube was vortexed for

30 sec. The tubes were then heated to 85 °C for 90 min and cooled to room temperature. Saturated (26%) NaCl, 1.5 ml, was added with 1 ml hexane and vortexed for two min. The tubes were centrifuged at 4,000 rpm for four mins and the top (organic) phase was then removed with clean glass Pasteur pipettes to a new tube. The remaining sample was then mixed with one ml hexane and extracted twice more. Once all three hexane layers were transferred to a new tube, the solvent was evaporated to dryness under nitrogen at 23 °C (room temperature). Samples were then resuspended in 200 µl hexane and transferred to glass sampler vials.

2.9.2 Quantification of FAMEs by gas chromatography-flame ionisation detector (GC-FID)

Fatty acid quantification was performed through quantification of FAMEs prepared through direct transmethylation and stored in hexane. A FAME standard in hexane and a blank sample (hexane) was used to account for contamination before each run. Fatty acid species were separated by the use of a GCMS-QP2010 SE (Shimadzu) and a BPX70 column of 120 meters, I.D 0.25 mm and 0.25 mm film thickness (SGE, Melbourne, Australia) column. Hydrogen gas was used as a carrier, generated by a CFH200 hydrogen generator (Peak Scientific). The injector was heated to 225 °C, and the transfer line was maintained at 250 °C. Samples were injected at 50 °C, and after 90 sec the temperature was raised to 150 °C at a rate of 15 °C per minute. Samples were then heated to 250 °C at a rate of 6 °C per minute and retained at this temperature for 3 min. Shimadzu software was used in conjunction with the GCMS-QP2010 SE to perform crude analyse and export relevant chromatogram data to an Excel sheet for further analysis. Each sample was then normalised to the C17:0 internal standard accounting varying conversion efficiencies from FA or TAGs to FAMEs. The internal standard and the FAME standard were used to calculate and give absolute values of µg/ml for each strain. Finally, the data was converted into graphical formats with GraphPad Prism 7 software.

Chapter 3 Results and Discussion

The aim of this project was to create a workable platform, in which SCRaMbLE technology coupled with a high throughput robust screening method can be exploited to generate novel oleaginous yeast strains. The ultimate goal is to secure strains with increased palm oil production.

3.1 Choosing a yeast strain for gene expression and SCRaMbLE

One of the main criteria for any study addressing gene expression is choosing the recipient strain for the genes of interest. In this case, the primary requirement is that the host strain is compatible with the SCRaMbLE. There are now several *S. cerevisiae* strains available that carry synthetic chromosomes containing loxP sites allowing SCRaMbLEing (Table 1). From the two *S. cerevisiae* strains, SynXIV and SynXVI developed at Macquarie University within the Sc2.0 project, the SynXIV strain is currently stable and ready for further manipulation. The SynXIV strain will facilitate a gene flanked by loxP sites to be intergraded into the genome up to 260 times. Therefore, several copies of a gene coupled with a strong promoter, SCRaMbLEd in, should produce a measurable phenotypical change. While the native fatty acid synthesising gene *ACC1* is located on the synthetic chromosome (XIV), it is not flanked by loxP sites and therefore SCRaMbLE should not pose a problem.

Table 7. Features of the available Sc2.0 chromosomes [91].

Number	WT size	SYN size	No. of stop codon swaps	No. of loxP sites added	Bp of PCRTag recoded	Bp of RE sites recorded	No. of tRNA deleted	Bp of tRNA deleted	Bp of repeats deleted
Chr03	316617	272195	44	100	5272	250	10	794	7358
Chr06	270148	242745	30	69	4553	369	10	835	9297
Chr09	439885	405513	54	142	7943	436	10	736	11632
Chr14	**784333**	**104096**	**96**	**260**	**13329**	**1113**	**14**	**1152**	**5115**
Chr16	948066	902994	127	334	15493	1374	17	1338	10048

3.2 Designing the plasmids containing the four selected genes encoding fatty acids

The plasmids for this work were built using pTEF1-*ScpC*-TSynth, a modified pRS416 plasmid as the backbone (Fig. 4). The plasmid backbone is a classic yeast cloning vector, pRS416 with a centromeric origin of replication and URA3 gene as the transformation

marker. The plasmid contains a strong constitutive *TEF1* promoter and a synthetic terminator TSynth. The other elements of the plasmid are the bacterial ampicillin resistance gene and the bacterial origin of replication for propagation of the plasmid in *E. coli*. The pTEF1-*ScpC*-TSynth plasmid was modified to include *S. cerevisiae* fatty acid synthesis genes *ACC1, ACS1, FAS1* and *FAS2* separately. These genes were inserted into the plasmid backbone replacing the *ScpC* coding sequence in the plasmid (Figs. 4- and 5). The plasmid backbone contains two loxP sites (section 1.6.1 Introduction) flanking the regions outside the *pTEF1* promoter and the *Tsynth* terminator, enabling rearrangement of the genetic material between the loxP regions. All four fatty acid encoding genes are expressed under the strong *TEF1* promoter.

To use the pTEF-ScpC-TSynth plasmid backbone to construct the four plasmids specific primers needed to be designed. Geneious software was used to construct a virtual plasmid for each gene (Geneious) [84]. Primers 3-10 (section 2.2 materials and methods) were designed to enable cloning of the genes (*ACC1, ACS1, FAS1* and *FAS2*) from the genome into the plasmid backbone (Figs. 4 and 5). Additionally, primers 11-18 (section 2.2 materials and methods and Fig. 5) were designed to be used with the Gene Sfi forward and reverse primers

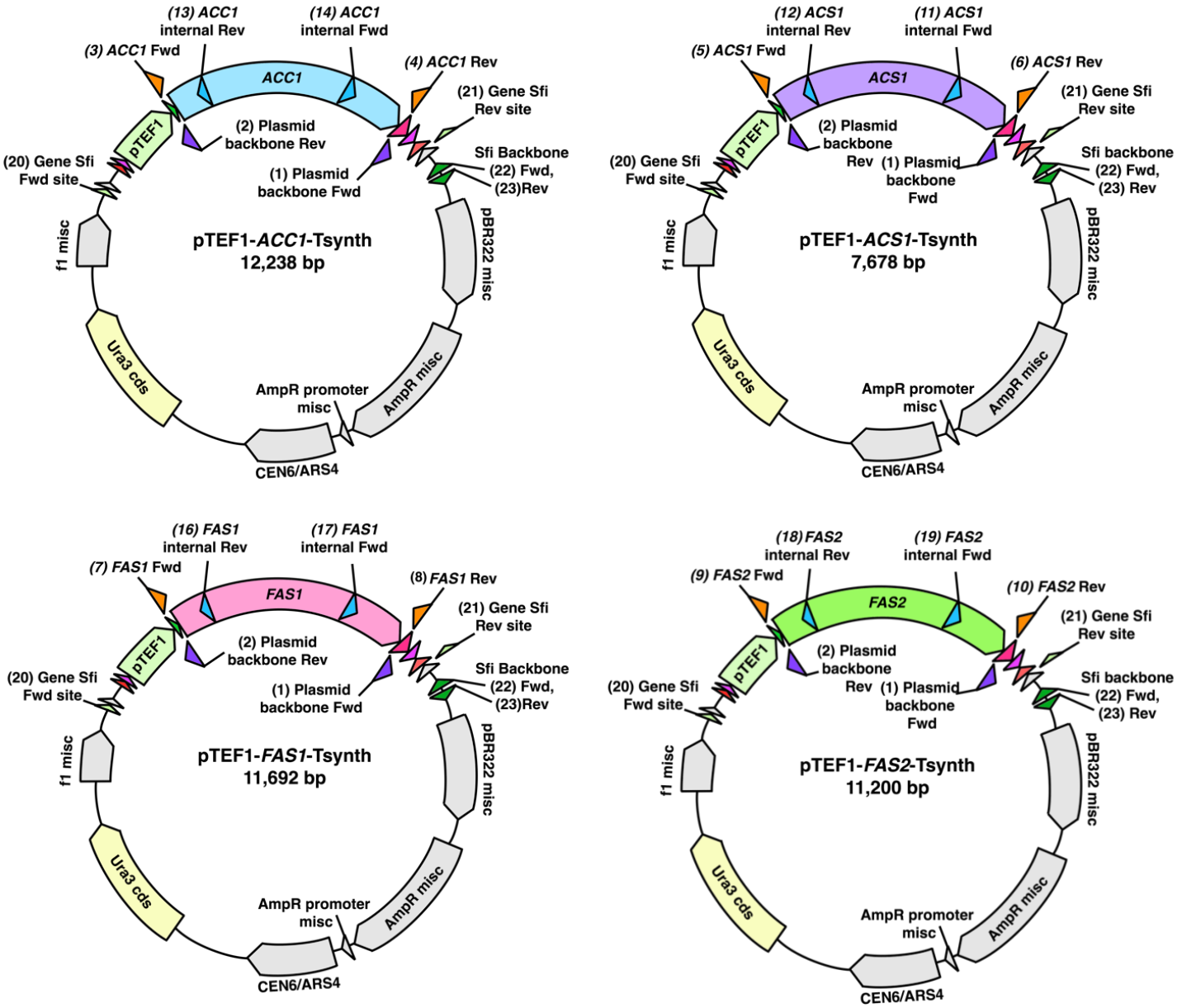

Fig. 5*. The designed plasmids that feature one of the four fatty acid synthesis genes each. These plasmids contain exactly the same backbone (from primer location 1 to primer location 2) as the original pTEF-ScpC-TSynth plasmid. The plasmids were used to design the primers 3 to 22 to enable assembly of the genes into the backbone or easier PCR confirmation or to enable the plasmids to be joined together with a SfiI restriction digest. Note, the plasmids are not drawn to scale to keep visualisation simple, this includes the primer binding sites and sizes of each gene.*

19 and 20 (section 2.2 materials and methods and Fig. 5) to enable easier PCR confirmation as this reduced the size of the PCR amplicons to 629-779bp.

3.3 Cloning *ACC1, ACS1, FAS1* and *FAS2* genes and the plasmid backbone for yeast and Gibson assembly

To build the four new plasmids the required building blocks needed to be isolated and put together. Towards this end, the FA encoding genes *ACC1, ACS1, FAS1* and *FAS2* were amplified from the *S. cerevisiae* BY4741 genome and the pTEF1-ScpC-TSynth plasmid backbone (available in house from Dr Tom Williams) was amplified from the pTEF1-ScpC-TSynth stock plasmid (Fig. 6) using PCR. Compatible overlapping regions were included for assembling the building blocks (section 2.2 materials and methods).

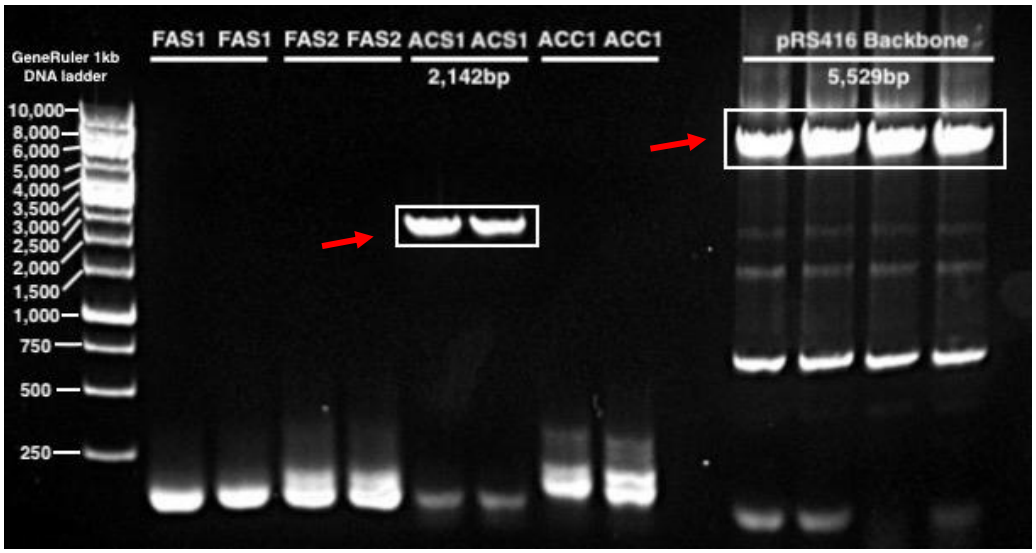

Fig. 6*. Successful PCR amplification of the ACS1 gene from the BY4741 genome and the pTEF1_ScpC_Tsynth plasmid backbone from the pTEF1_ScpC_Tsynth template. Shown by the red arrows are the amplicons for ACS1 (2,142bp) and the pTEF1-ScpC-TSynth backbone (5,529bp) at their expected sizes.*

ACS1 and the pTEF1-ScpC-TSynth plasmid backbone were successfully amplified by the first attempt (Fig. 6). However, initial attempts to isolate the *ACC1, FAS1 an FAS2* genes with generic PCR parameters were unsuccessful. Several PCR conditions, DNA polymerases and genomic DNA concentrations were then explored (section 2.3.1 Materials and Methods). The DNA polymerase LongAmp was found to be best suited to replicating the longer *ACC1, FAS1* and *FAS2* genes. Accordingly, the PCR conditions were appropriately adapted to amplify long DNA segments, which included extending the elongation time leading to successful amplification of the *ACC1, FAS1* and *FAS2* genes (Fig. 7).

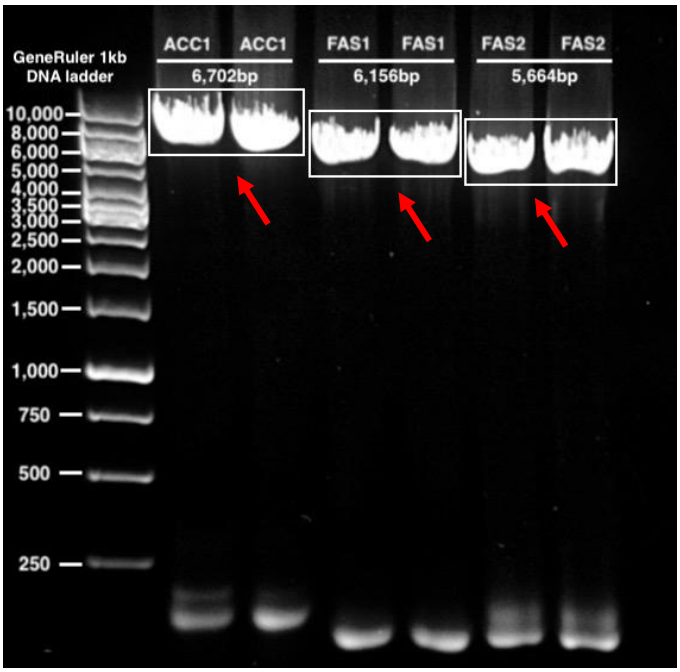

***Fig. 7**. Successful PCR amplification of ACC1, FAS1 and FAS2 gene sequences from the S. cerevisiae BY4741 genome. Shown by the red arrows are the amplicons for ACS1 at their expected sizes of ACC1 6,7020bp, FAS1 6,156bp and FAS2 5,664bp indicating their successful amplification.*

Once all the building blocks were cloned with compatible overlapping regions, they could be assembled together. To perform this task, yeast assembly was chosen.

3.3.1 Yeast assembly

To generate recombinant SynXIV strains with plasmids containing the genes *ACC1, ACS1, FAS1* and *FAS2*, the host strain SynXIV was separately transformed with one the four genes and the pTEF1-ScpC-TSynth plasmid backbone sharing an overlap of about 25 bp to provide homology allowing the two building block to self-assemble via yeast *in vivo* homologous recombination. This process was performed in four separate reactions to generate the four new strains: SynXIV-*ACC1,* SynXIV-*ACS1,* SynXIV-FAS*1* and *SynXIV-FAS2*. The newly formed strains harbouring recombinant plasmids were immediately plated onto selective uracil drop out medium ensuring only strains with a correctly assembled plasmid with the URA3 gene could grow. The same assembly method was applied to a negative control containing only the plasmid backbone to provide information on the assembly efficacy. The results suggested that nine out of ten colonies on each plate contained a successful plasmid assembly. Ten colonies were then picked from each plate and analysed by PCR using the specific primer 19 with the respective reverse primer for each gene (primers 12, 14, 16 or 18)

which amplified from the backbone to the gene (insert) (section 2.2 Materials and Methods). PCR primers were designed so that amplification could only occur if the plasmids are assembled in the correct orientation. Strains which passed the first round of screening were tested again with the same method several days later (Figs. 8 and 9) to confirm the presence of the correct plasmid before glycerol stocks were made. Recombinant strains each containing a correctly assembled plasmid with one of the four FA encoding genes were then used to develop a high lipid screening protocol.

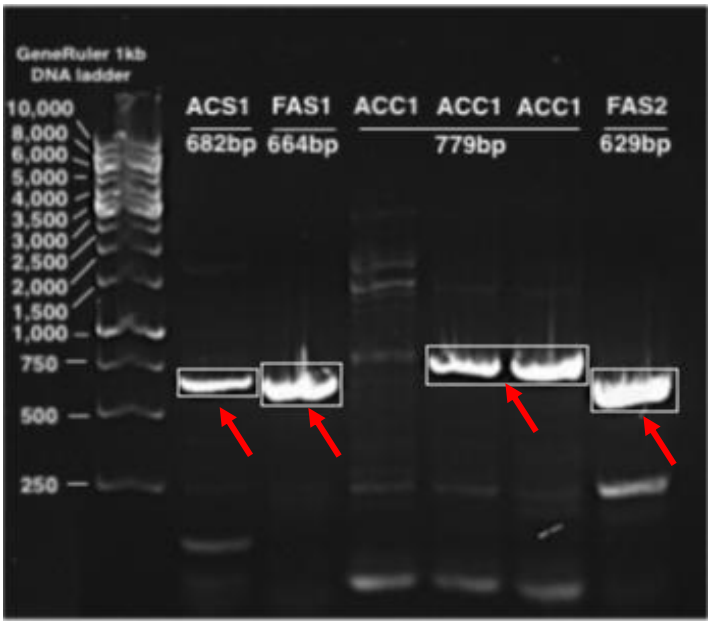

Fig. 8. Checking the created plasmids from yeast assembly with PCR using primers designed to bind to the existing backbone and the newly inserted gene (Fig. 5). PCR amplicons can occur only if the plasmid is assembled in the correct orientation. One colony for each strain is shown with the amplicons at the expected size confirming the correct assembly of all four plasmids. The ACS1 amplicon is shown at the expected size 682bp, the FAS1 amplicon here is 664bp, the multiple ACC1 amplicons are shown here at 779bp and the FAS2 amplicon is also shown at 629bp. All the amplicons are at their expected sizes susjusting the correct construction of the plasmids.

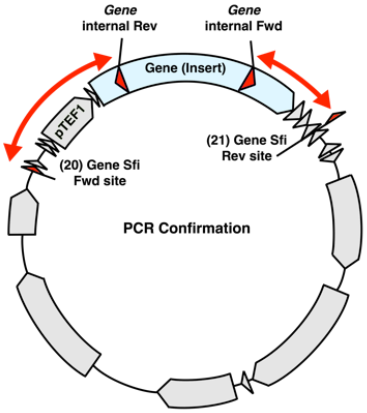

Fig. 9. A simplified plasmid example showing how PCR was used to confirm the correct assembly of each recombinant plasmid. Specific primers allowed amplification for each gene spanning from the plasmid backbone to the gene (insert). The red arrows represent the location of the PCR amplicons.

3.3.2 Gibson assembly

Since the chosen pTEF1-ScpC-TSynth plasmid is a low copy number plasmid in yeast, it was difficult to extract plasmid DNA at high quantities from the new recombinant strains. Although not affecting this project, low availability of plasmid DNA would hinder using these plasmids in the future work as they cannot be further transformed into different strains and plasmid stocks cannot be made and stored. To overcome this obstacle, separate amplicons representing each gene and the backbone of the pTEF1-ScpC-TSynth plasmid were also assembled *in vitro* using Gibson assembly (Fig. 10) and cloned into *E. coli* to generate plasmid stocks for future work. Similarly, to the yeast assembly, the Gibson assembly was

examined by screening several clones for each of the four plasmids. Correct assembly was confirmed by PCR and DNA stocks were made.

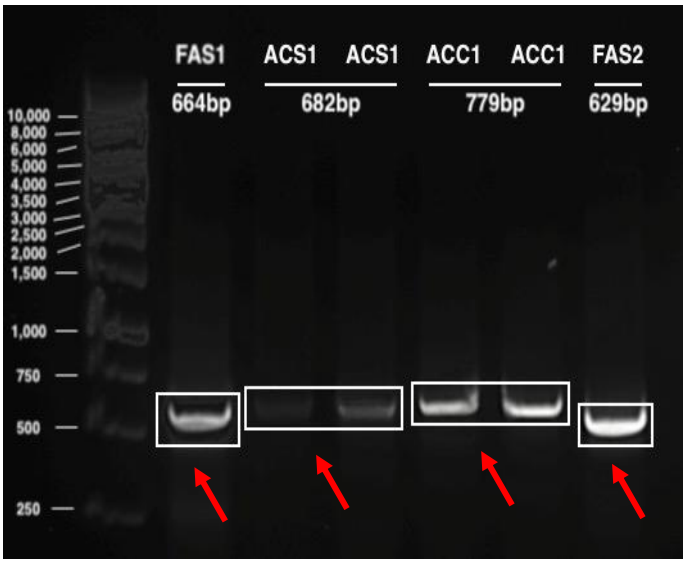

***Fig. 10**. Checking the plasmids made by Gibson assembly with PCR using primers designed to bind to the existing backbone and the newly inserted gene. At least one colony for each strain is shown (red arrows) with the amplicon at the expected sizes with the ACS1 amplicon is shown at the expected size 682bp, the FAS1 amplicon here is 664bp, the multiple ACC1 amplicons are shown here at 779bp and the FAS2 amplicon is also shown at 629bp confirming the correct assembly of the plasmids.*

3.4 Development of a screening protocol for high lipid producing strains

A robust, efficient and preferably high throughput screening protocol is essential for screening new strains producing increased amounts of fatty acids, especially after SCRaMbLE. This requires a correct combination of the cell cycle, lipid stain and a machine capable of detecting changes in the amounts of fatty acids produced.

3.4.1 Establishing a staining protocol with Nile red for the SynXIV strain

To measure fatty acid content within the cell, the lipid stain Nile red was employed as it has been shown to be capable of staining neutral lipids (TAGs) Fig. 11 [92]. To determine a suitable concentration of Nile red for flow cytometry used here for detection, three different concentrations of the stain were applied to the SynXIV strain containing no recombinant plasmids. The SynXIV strain was grown for 72 h in liquid YPD medium to boost fatty acid production and 60 µg/ml (1x) Nile red was used as a starting point based on an earlier study [89]. The criterion was that Nile red needed to be able to measure TAGs accurately while without saturating the detector of the flow cytometer. Two other concentrations, 0.5x (30 µg/ml) and 2x (120 µg/ml) of Nile red were also tested. The cells were grown for 72 h

ensuring the cell cycle had reached the stationary phase known to be the phase when lipids are accumulating in yeast [93]. After this, the cells were stained for five minutes, then washed twice in 1x PBS (section 2.81 Materials and Methods) and fluorescence measured on the Beckman Coulter CytoFLEX flow cytometer (CytoFLEX) with the laser excitation of 488, and emission at 580/30 nm (FL1-A :: TAGS-A) (Fig. 11).

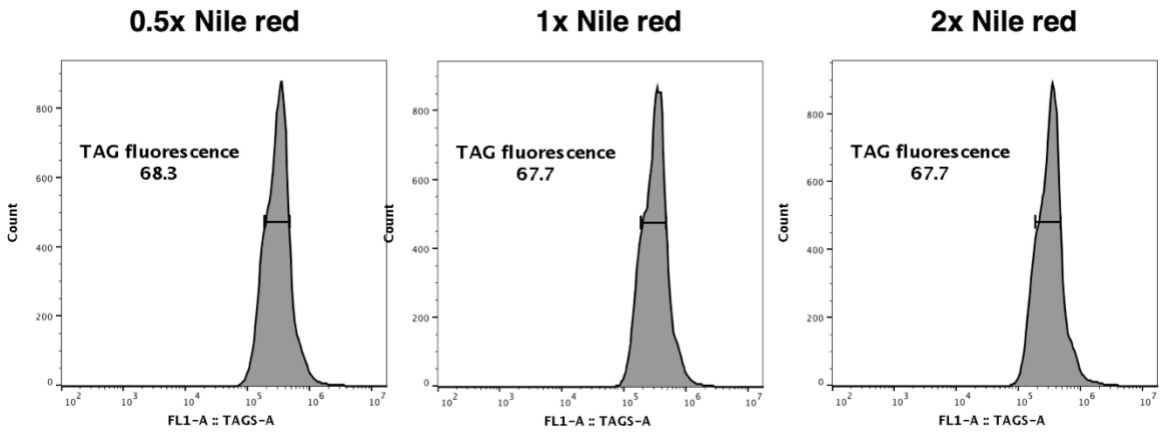

***Fig. 11**. Fluorescence from Nile red detected in SynXIV with TAGS-A. The different concentrations of Nile red 0.5x, 1x, 2x are shown left to right. The coordinates given in the figure represent the percentage of the population expressing TAG fluorescence to visualise differences between the populations. From initial visualisation no differences were determined. The mean area of 0.5x was 354182, with 1x at 355254 and 2x at 356927. Although, because only one value was measured from each concentration no statistical analysis was performed. From visualising the TAGS-A histograms and single TAG-S areas no differences were determined between the mean area.*

The TAGs-A (TAG Area) mean fluorescence values were then compared across the different Nile red concentrations, and no significant difference in intensity was determined. This suggests that any of the three Nile red concentrations used here were suitable for the analysis of the fatty acid content by flow cytometry as set out. The 1x Nile red concentration (60 µg/ml) was chosen for further experiments.

3.4.2 Proving flow cytometry can measure TAG fluorescence

After establishing a suitable Nile red concentration to detect lipids in the SynXIV strain which will host the added FA encoding genes, a series of experiments were set out to test if different concentrations of lipids were detectable by the flow cytometer. As lipid production in yeast is affected by the availability of carbon source (here glucose) [94, 95], it is possible to produce high and low amounts of lipids by regulating the amount of glucose in the culture. Towards this end, the SynXIV strain was grown in YPD medium with 0%, 0.5%, 1% or 2% (w/v) added glucose for 72 h at 30°C. The cells were then stained with Nile red as above and

fluorescence excited at Ex 488nm and measured at Em 584/42 nm on the Cytoflex flow cytometer (Fig. 12).

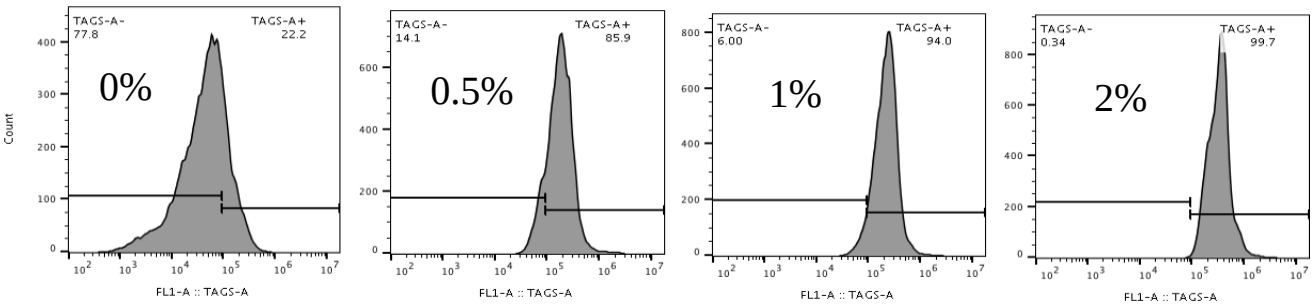

***Fig. 12**. The four different concentrations of glucose increasing from 0%, 0.5%, 1% to 2% left to right. A reference point was chosen (10^5 TAGS-A) to compare samples. At 0% glucose only 22.2% of the population is greater than the reference point, this is expected as the cell are not able to store TAGs without a carbon source. At 0.5% glucose 85.9% is greater and at 1%, 94% is greater. At 2% glucose 99.7% of the population is greater than the reference point. 20,000 cells were captured for each sample.*

Shown in Fig. 12 are the yeast populations grown on different amounts of glucose, stained with Nile red and their fluorescence measured at 584/42 nm. There seems to be a positive relationship between the level of glucose and increased fluorescence, no statistical analysis was performed. To ensure the change in fluorescence represented a genuine increase in the concentration of fatty acids stored in TAG form, fatty acids from cells from each culture with a different amount of glucose were extracted and converted into fatty acid methyl esters (FAMEs) for GCMS analysis (Fig. 13).

The lipid profile of SynXIV grown in different concentrations of glucose (Fig. 13) correlated with the levels of fluorescence displayed in Fig. 12 suggesting the change in fluorescence was due to the increased concentrations of stained TAG molecules.

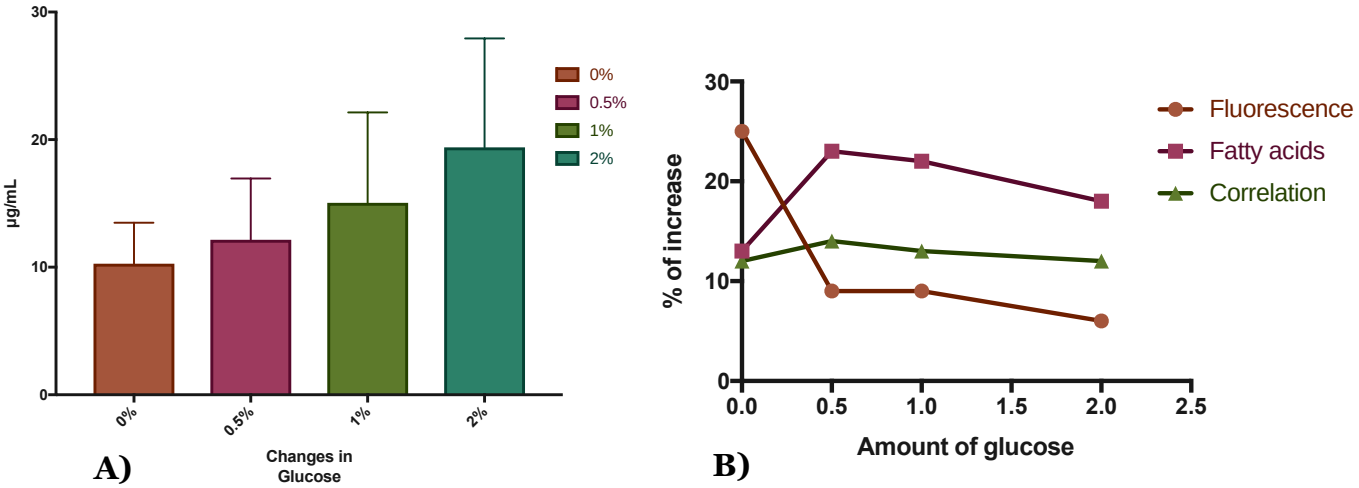

Fig. 13. ***A)** The total combined of C16:0, C16:1 C18:0 and C18:1 fatty acid profile of SynXIV grown under different concentrations of glucose. At 0% glucose, 12 µg/ml of fatty acids were produced. At 0.5% glucose, 17µg/ml of fatty acids were produced. At 1% glucose 22µg/ml of fatty acids were produced and at 2% glucose, 28µg/ml of fatty acids were produced. This suggests an increase in glucose results in an increase in the amount of fatty acids produced. **B)** The fluorescence is compared to the fatty acid profiles by the changing levels of glucose within the medium. The x axis shows the changes in glucose with the fluorescence shown by the circles and the fatty acids shown by the squares. The results suggest there is a correlation between the fluorescence and the fatty acid profiles confirming the FACs is able to distinguish difference lipid profiles within the cell.*

This result also suggests the flow cytometer is sensitive enough to detect a 2 μg/ml change in fatty acid content between strains. Thus, flow cytometry is capable of for detecting small increases in the amount of TAGs which can be used for screening novel yeast strains created through SCRaMbLE, also allowing preliminary quantification of fatty acids within cells. The work within the host SynXIV strain has also provided a baseline for comparison of the fatty acid content of different strains.

3.4.3 Establishing a flow cytometry sorting protocol

Before SCRaMbLE could occur, a gating protocol for the SynXIV, Syn-*ACC1*, Syn-*ACS1*, Syn-*FAS1* and Syn-*FAS2* needed to be developed (Fig. 14). Creating gates involved setting certain parameters on the flow cytometer according to which the cells are selectively sorted in real time. If a cell is within the predetermined gates (Fig. 14) it will be sorted into a collection tube or agar plate and interrogated further.

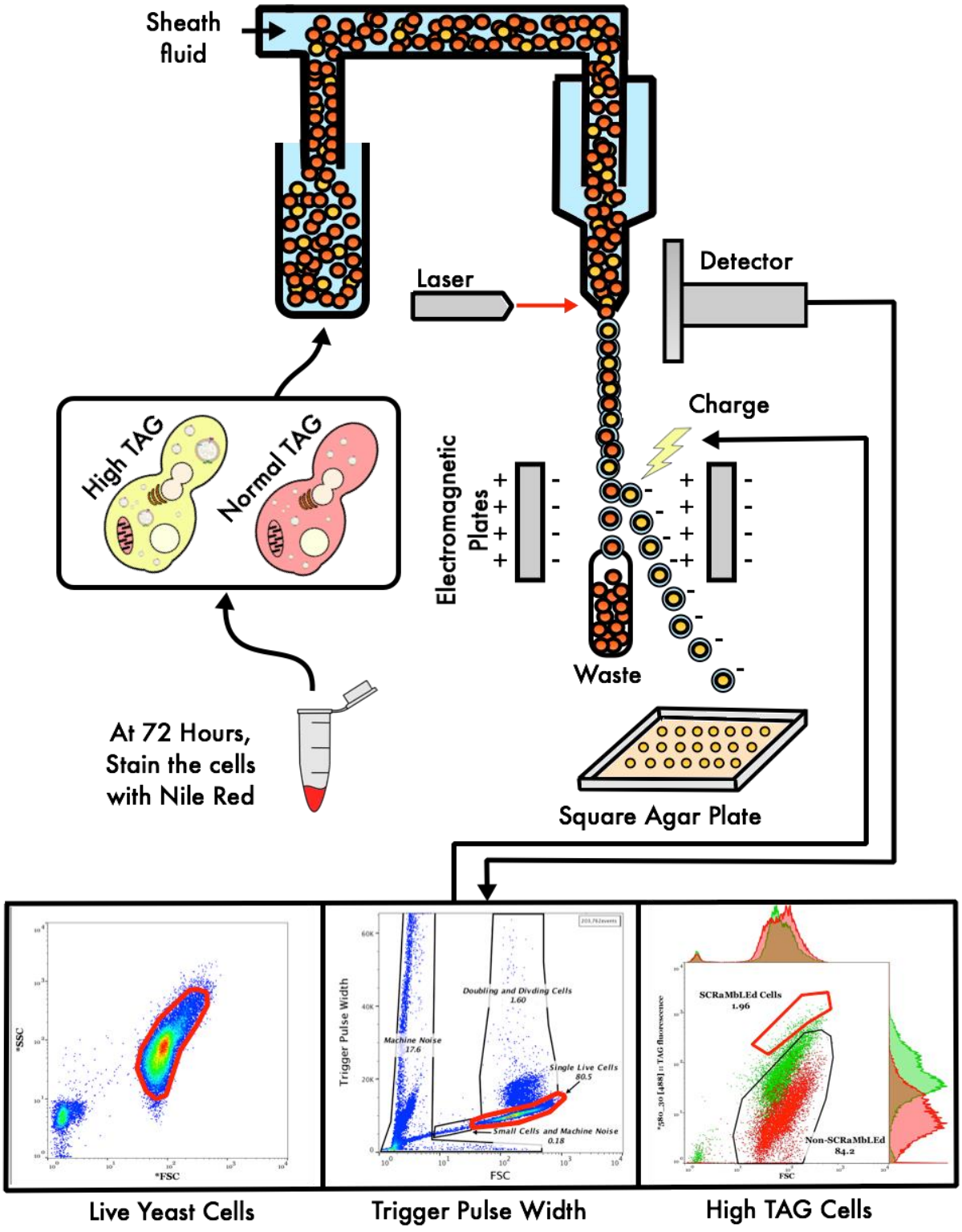

***Fig. 14**. Screening protocol for yeast cells producing fatty acids. Yeast cells will be grown to the stationary phase (72 h) and stained with Nile red. Next, the flow cytometer will be calibrated. Each different yeast strain will then be processed one at a time through the flow cytometer with fluorescence-activated cell sorting (FACS). The cells are then subjected to the three gates. First, for a cell to be sorted it must be within the "live cell gate" shown in red, second, the cell must be within the "trigger pulse width" gate shown in red. Finally, the cell must be within the "high TAG cell" gate shown in red. Only when a cell is determined to be within all three gates is it then sorted onto the agar plate for further work. If a large number of colonies resulting from one round of sorting, then another round may be employed with stricter gating of the "High TAG cells" gate.*

To establish the gating protocol, the cells from each strain harbouring a recombinant plasmid were grown to stationary phase (72 h) in 10 ml URA- minimal medium and SynXIV in minimal medium with amino acid supplements, stained with Nile red, and then loaded into the BD Influx flow cytometer. Three gates were applied according to the method in Fig. 13. First, the live cell gate was applied to ensure the populations with the correct forward and side scatter respective to live yeast cells. Second, the populations were gated by a "Trigger Pulse Width". Finally, a normal TAG gate was applied to each respective sample to ensure a TAG baseline for these strains was set for further screening in the future (section 3.2.5 Materials and Methods).

The envisaged workflow is as follows. If a cell is successfully detected within all three gates, a negative charge will be applied to the water droplet carrying the cell (Fig. 13). When passing the electromagnetic plates, the negatively charged water droplets are drawn towards the positive electromagnetic plate separating it from the mainstream. A square agar plate calibrated to accept either 96 or 384 cells will then move into place automated by the machine to separate each cell into a defined position. The plates containing cells sorted according to the gates will be incubated for three days at 30°C to recover the newly sorted strains. Depending on the number of colonies, the cells will be put through another round of sorting with a stricter gate applied to the “High TAG Cells”. Capture of the highest lipid producing strains may thus take several rounds of sorting. Finally, strains with the most intense TAG fluorescence will then be subjected to fatty acid analysis.

3.4.4 Sorting single cells with trigger pulse width gating

The trigger pulse width (TPW) gate was applied to ensure “Single Live Cells” (Fig. 15) are sorted and not cells about to divide or cells with abnormal shape and size. Dividing cells and abnormal cells could have higher TAG fluorescence simply due to their cell cycle and size, and not because of their individual ability to produce high amounts of TAGs. The TPW measures the particle size and intensity as it passes in front of the laser. As different shapes result in different TPWs, the TPW allows for accurate separation of detected events from the machine noise, contaminations and doubling cells or abnormal cells (Fig. 15). In this sorting protocol, doubling cells pose the most significant problem as they will result in a false high neutral and polar lipid fluorescence. Taking these cells for further analysis would create unnecessary additional work. Applying TPW ensures only normal sized single cells (based on the SynXIV strain) with genuine high TAG fluorescence will be sorted. One potentially negative consequence of strict TPW gating is that abnormal or size-wise mutated cells will

be ignored even if they genuinely produce high TAGs. However, because SCRaMbLE here will be performed in the SynXIV strain with only one synthetic chromosome, the chances of developing physically abnormally shaped cells from SCRaMbLE is small. If a cell is mutated enough to be detected outside the TPW gate, the mutation it is likely to affect the cell negatively. For these reasons, it was decided to sort cells which resemble a normal yeast shape and produce high amounts of TAGs.

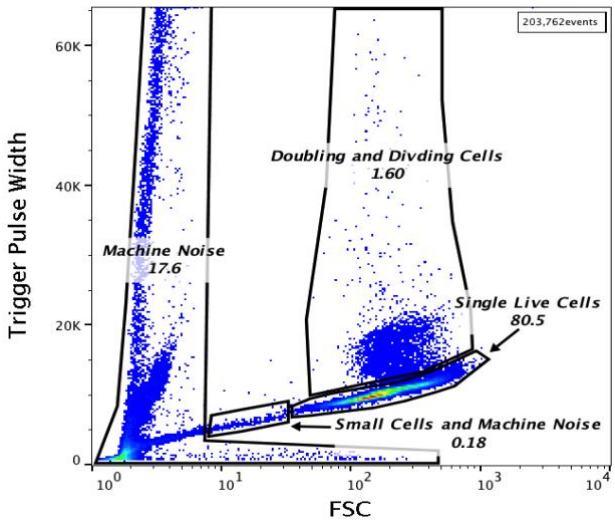

Fig. 15. *TPW distinguishing single live cells from the background noise. Each number shown represents the relative percentage of the total events recorded. For example, the events labelled as "Machine Noise" make up 17.6% of the total population and are a result of the high throughput process, or biofilm build up within the machine or random auto fluorescent particles. While the majority of the events are the single live cells (80.5%). A typical run would not include so much machine noise, but this sample was run for over 200,000 events with an intent to show the accumulation of machine noise usually unnoticed in shorter run experiments. If not gated properly undesired evens could be sorted preventing desired cells from being sorted.*

3.4.5 Establishing a fluorescence profile for each single recombinant strain

To determine a “baseline” of TAG fluorescence for each recombinant strain harbouring a fatty acid encoding gene, all strains were tested with gating in place (Fig. 16). The recombinant strains were grown in URA- minimal media with 2% glucose and SynXIV in minimal media with all amino acids added for 72 h at 30°C and stained with 1x Nile Red. Each sample was gated by “Live Yeast Cells”, “High TAG cells” and the “TPW” according to Fig. 14. It turned out that the Syn-*ACC1*, Syn-*FAS1* and Syn-*FAS2* strains showed an increase of TAGs when compared to the SynXIV strain, suggesting that expressing these genes under a strong constitutive *TEF1* promoter was enough to result in a shift in TAG fluorescence.

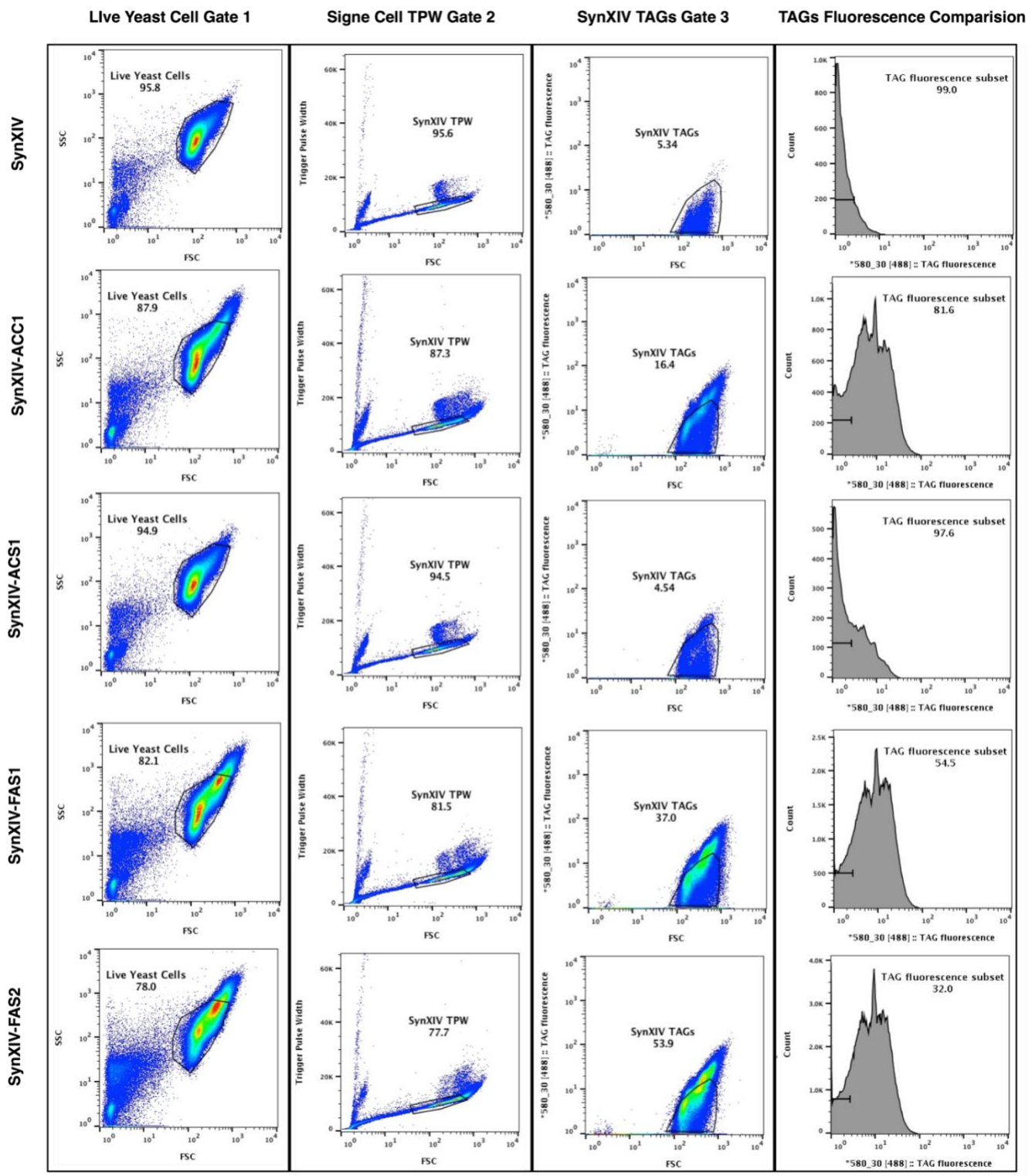

Fig. 16*. Forward and side scatter profiles of the different recombinant strains measured against the synthetic strain SynXIV (control). Measurement was performed at an Ex 488 Em 580/30 nm laser with 50,000 cells measure for each sample. The SynXIV strain was included to help determine how healthy cells would look like within the BD Influx. The same logic was applied to the TPW gate. The SynXIV TAGs gate was also applied to all strains. By comparing the strains, an increase in TAGs can be seen in Syn-ACC1, Syn-FAS1 and Syn-FAS2, with a small increase with Syn-XIV. The far-right column is a direct comparison of the TAGs shown through a histogram format of fluorescence of Nile red representing TAGs within the cell. Shown by the fixed point the TAG population increases in the recombinant strains.*

Instead, Syn-*ACS1* showed no significant change suggesting expression of *ACS1* alone either cannot result in the synthesis of increased amount of TAGs under these conditions or the *ACS1* gene is heavily regulated by other factors. The increased TAG fluorescence were directly compared to quantify the TAG fluorescence between the strains (Fig. 16). With a histogram representing TAG fluorescence a fixed point was chosen to account for 99% of the SynXIV. The recombinant strains were then compared to the SynXIV strain shown in table 8.

Table 8. TAG Fluorescence comparison between recombinant strains

Strain	% of TAGs higher than SynXIV
Syn-*ACC1*	>18
Syn-*ACS1*	>1.4
Syn-*FAS1*	>44.5
Syn-*FAS2*	>67

This suggests *ACC1*, *FAS1* and *FAS2* are producing much greater level fatty acid levels than the SynXIV. Although, to gain a deeper understanding a quantitative analysis of lipids/total fatty acids in these cells is needed.

3.4.6 The fatty acid profiles of all recombinant strains

To confirm that the observed changes were genuine changes in the amount of TAGs, fatty acid extraction and analysis was performed. In addition to providing quantification of the TAG content in the different recombinant strains, the analysis would also provide a baseline for the measurement of the increase or decrease of the lipid content of any strain resulting from SCRaMbLEing the four genes. The recombinant strains were grown in triplicate for 72 h at 30°C with 2 % glucose in a synthetic minimal medium without uracil. Direct transmethylation (section 2.9.1 Materials and Methods) was then performed to convert the TAGs into fatty acid methyl esters (FAMEs).

3.4.7 Fatty acid profiles of SynXIV, SynXIV-ACC1, SynXIV-ACS1, SynXIV-FAS1 and SynXIV-FAS2

To calculate the fatty acid profiles for each strain, a method was devised to enable an absolute calculation of the FAMEs measured at the GCMS detector in the form of a chromatogram and to back calculate the amount of fatty acids within the original culture. Firstly, to perform these calculations a known amount of FAMEs were run through the

GCMS with their chromatographic peaks measured. For this, a FAME standard comprised of C12:0, C14:0, C14:1, C16:0, C16:1, C18:0, C18:1, C18:2, C20:0 and C21:1 FAMEs. Because a pure FAME C17:0 sample was not available, the FAME standard was used to quantify the amount of C17:0 FAME within samples. This was done by producing a standard curve and calculating the position of C17:0 between C16:0 and C18:0 FAMEs. The amount of C17:0 FAME was then calculated based off the area under the curves of the C16:0 and C18:0 chromatographic peaks. Once this was achieved, the C17:0 internal standard was used in every sample to both calculate the conversion efficacy of fatty acids to FAMEs and provide a quantitative number (µg/ml) for the amount of fatty acids within 1 ml of culture. Following this, the samples were normalised by cell count. Shown in Fig. 17 are the relative fatty acid profiles of the strains harbouring recombinant plasmids and the SynXIV strain for comparison. /

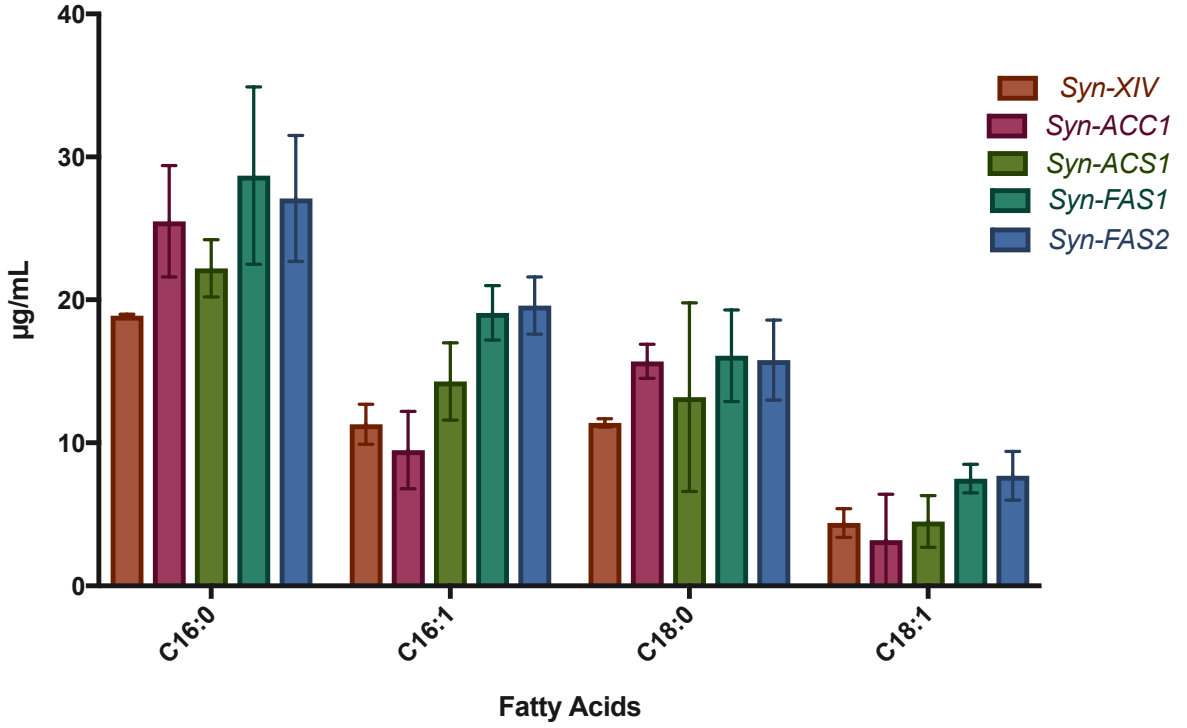

***Fig. 17**. The fatty acid profiles of the recombinant strains are shown and compared to the SynXIV strain. In all strains, there is more palmitic acid (C16:0) and stearic acid (C18:0) produced than palmitoleic acid (C16:1) and oleic acid (C18:1). It was expected from the flow cytometry fluorescence profiles that ACC1, FAS1 and FAS2 would have a higher fatty acid content than ACS1 and SynXIV. Here the same trend was observed with FAS1 showing the highest levels of fatty acids. All the recombinant strains showed a significant increase in fatty acid levels when compared to the parent SynXIV strain.*

The results in Fig. 17 show that the SynXIV strain had the lowest fatty acid composition followed by Syn-*ACS1* and then Syn-*ACC1*, Syn-*FAS2* and finally Syn-*FAS1*. Moreover, results shown in Fig. 16 verify the positive relationship between the amount of TAGs produced as indicated by flow cytometry and (Fig. 16) the actual fatty acid profile (Fig. 17).

3.4.8 Comparison of the fatty acid profiles between the SynXIV and BY4741 strains

The fatty acid profile of C16:0, C16:1, C18:0 and C18:1 of SynXIV was compared to that of the typical lab strain BY4741 to determine if the synthetic strain SynXIV had any inherent problems producing fatty acids. Both SynXIV and BY4741 were cultured in triplicate for 72 h at 30°C in YPD medium with 2% glucose. The samples were then normalised to an OD of 1.0 and the cells counted with a haemocytometer to enable the final FAMEs to be also normalised by cell count. A C17:0 heptadecanoic acid internal standard was used to account for conversion efficiency of the direct transmethylation method and to quantify the fatty acid content. C17:0 was chosen for this project as the yeast fatty acid synthesis pathway cannot produce odd numbered fatty acid chains. Therefore, by using a C17:0 internal standard the C16 to C18 results were not affected. The results shown in Fig. 18 conclude the synthetic strain does not have any significant defects preventing it from synthesising the C16:0, C16:1, C18:0 or C18:1 fatty acids.

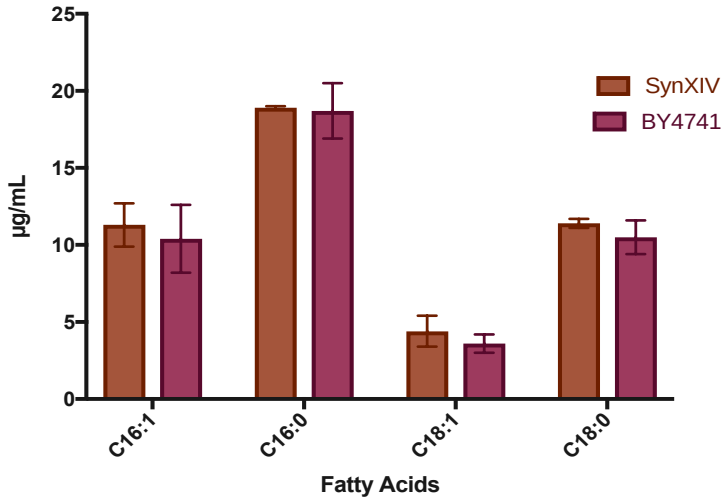

***Fig. 18**. A comparison of the fatty acid profiles of a laboratory yeast strain BY4741 and the synthetic strain SynXIV. Shown in orange are the amount of fatty acids C16:0, C16:1, C18:0 and C18:1 from SynXIV in µg/ml. In red are the equivalent fatty acid amounts from BY4741. There is no considerable difference in the ability between the strains to produce the featured fatty acids.*

By the same token, the SynXIV strain provides an adequate starting point for strain development with SCRaMbLE to produce yeast strains with improved fatty acid content and ultimately, an alternative source of palm oil which is rich in palmitic (C16:1) and oleic acid (C18:1).

3.5 SCRaMbLEing the recombinant strains

Once the C16:0, C16:1, C18:0 and C18:1 fatty acid profiles of each strain harbouring a recombinant plasmid and SynXIV without a plasmid was determined, the strains were ready

to be SCRaMbLEd. Towards this end, the strains were first transformed with the Cre recombinase plasmid containing *His3* prototrophy marker enabling selection of the transformants [96] (section 2.2 materials and methods).

3.5.1 SCRaMbLE Killing Assay

Amongst other outcomes, long SCRaMbLEing periods and results in synthetic lethal fitness defects due to undesirable recombination events, which can result in cell death [77]. Lethality increases with SCRaMbLE duration. Following from this, the efficiency of SCRaMbLE can be determined by performing a killing assay. The assay carried here involved Syn-*ACS1*, Syn-*ACC1*, Syn-*FAS2*, Syn-*FAS1* and SynXIV cells under the following conditions: SCRaMbLE induced and not induced by the Cre recombinase, tracked over nine hours (section 2.7.2 Materials and Methods). After counting the colonies from each SCRaMbLEd recombinant strain, a clear difference was found between the induced and not induced populations (Fig. 19).

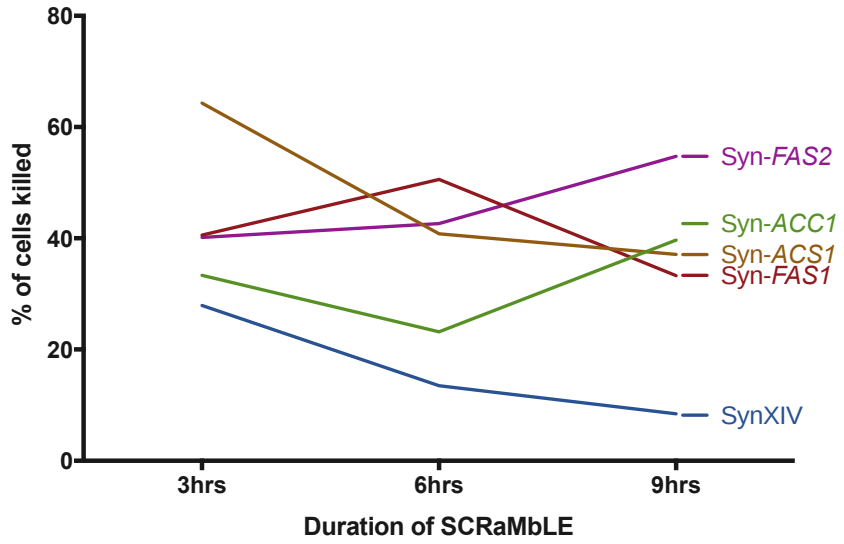

***Fig. 19**. The killing rates of each separate SCRaMbLE experiment over nine hours. The percentage of killing decreased in SynXIV, Syn-ACS1 and Syn-FAS1 over the nine-hour period suggesting SCRaMbLE has not occurred while the killing percentage in Syn-ACC1 and Syn-FAS2 following nine hours of SCRaMbLE increased as expected. This suggested the number of lethal genetic recombination's had increased causing cell death the longer SCRaMbLE was induced.*

As shown in figure 16, only Syn-*ACC1* and Syn-*FAS2* cells were increasingly killed over the nine-hour period suggesting that they had been SCRaMbLEd. On the other hand, based on this assay, SynXIV, Syn-*ACS1* and Syn-*FAS1* did not appear to be SCRaMbLEd. Some reasons for this may be that the strains had lost the Cre recombinase encoding plasmids or SCRaMbLE in these strains may have promoted genetic recombination that did not result in the killing of cells.

3.5.2 SCRaMbLEd Strains

The killing assays suggested that SCRaMbLE had occurred at least in Syn-*ACC1* and Syn-*FAS2*. The SCRaMbLEing rate is limited by the Cre recombinases ability to splice DNA in or out of the genome. Nine-hours of SCRaMbLE should result not only close to the maximum number of synthetic lethal combinations but also the greatest number of unique genomic combinations. For this reason, all strains were SCRaMbLEd for nine hours, then recovered for three days and sorted (Fig. 20)

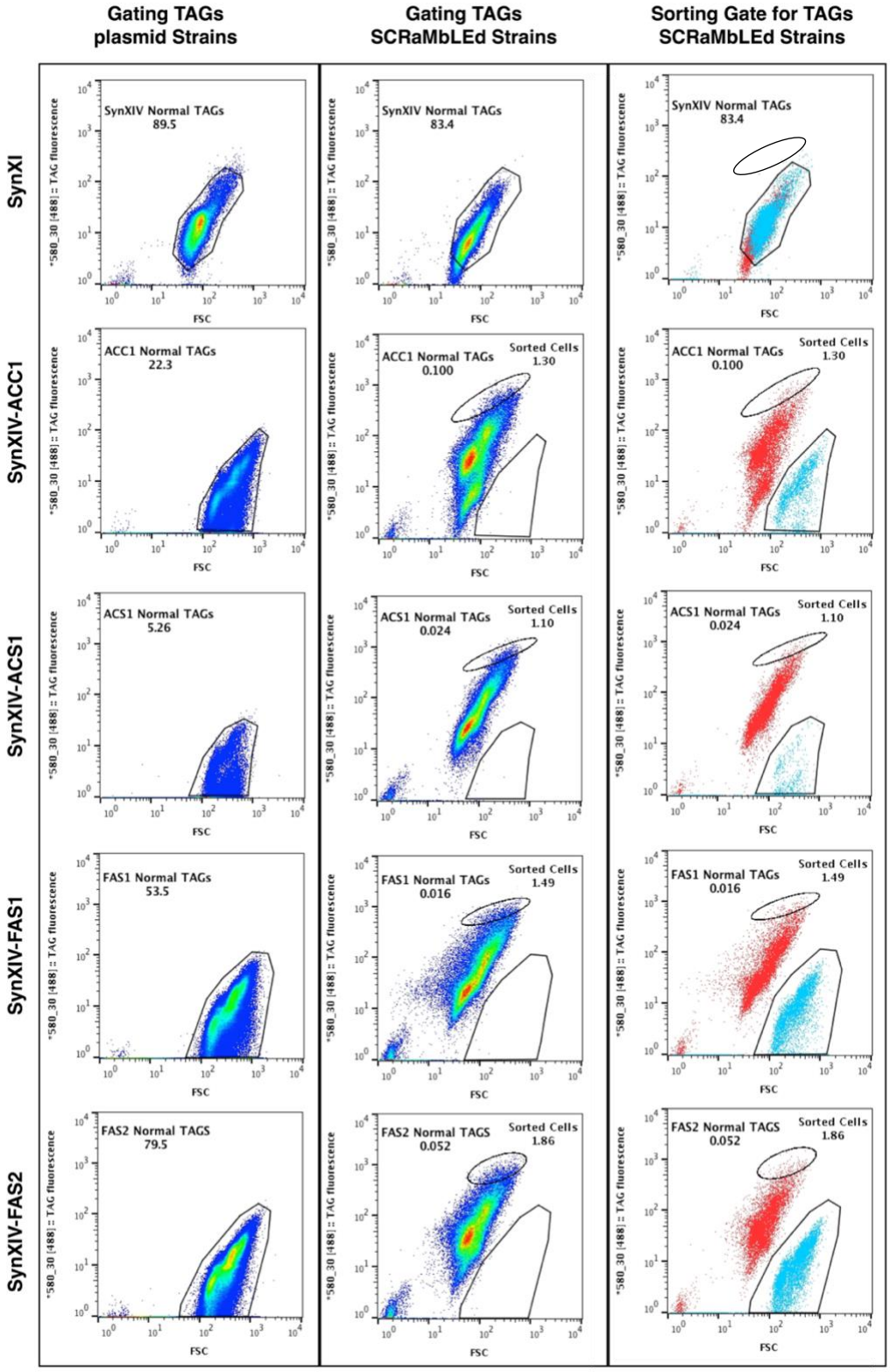

Fig. 20*. Gating the SCRaMbLEd strains and sorting high lipid cells. Similar to previous gating outlined (section 3.4.5) the SynXIV was used to gate both live yeast cells and single yeast cells, not shown. The recombinant strains (here are called plasmid strains) were used to create gates around each strain and its normal TAG fluorescence profile (Gating TAGs plasmid strains). Once this was done the SCRaMbLEd strains were viewed and compared to the non-SCRaMbLEd recombinant strains (Gating TAGs SCRaMbLEd strains). Next the two groups for each strain were plotted onto the same graph to show the difference between the TAG profiles (Sorting Gate for TAGs SCRaMbLEd strains). As seen in the column on the right all the SCRaMbLE cells (red) are higher on the TAG axis than the non-SCRaMbLEd cells. This suggests there is an increase in TAGs. Next a new gate was applied to the SCRaMbLEd populations and the top 2% of the TAG producing cells were sorted. 50,000 events were captured for each sample.*

The parent SynXIV strain was used to determine the "live cell gate" and the "TPW gate". The "high TAG cells" gate was applied to each separate SCRaMbLEd sample. This gate was determined first by comparing the non-SCRaMbLEd strains to the SCRaMbLEd ones. To see if there was a difference the two populations were compared on the same density plot to see if there is, firstly, a difference in the TAG fluorescence between the two samples (Fig. 20). Once a difference was confirmed, a new gate was applied to sort only those cells with the highest TAG fluorescence. This resulted in about the top 2% of each sample being sorted. By applying the three levels of gating, only single cells that were consistently sized and shaped like the SynXIV strain and that were producing higher levels of TAGs were selected (Fig. 20). Ninety-six events (cells) from each SCRaMbLEd strain including the SCRaMbLEd SynXIV were plated onto a square YPD agar plate and incubated for three days at 30°C. Unfortunately, only one colony from the SCRaMbLEd SynXIV, *ACC1* and *FAS2* each was recovered, and no colonies appeared on *FAS1* or *ACS1* plates. This suggests a serious survival issue, as 96 colonies were expected to grow. The SynXIV, *ACC1* and *FAS2* colonies were then named Syn-sXIV, Syn-s*ACC1* and Syn-s*FAS2* and their fatty acid content was analysed to determine if the sorting process was robust enough to select for high lipid producing SCRaMbLEd strains.

3.5.3 Fatty acid profiles *of* Syn-sXIV, Syn-s*ACC1* and Syn-s*FAS2*

To compare the amounts of fatty acid produced in strains harbouring the recombinant plasmids to the SCRaMbLEd strains, the same growth conditions would be needed. This was problematic as following SCRaMbLE, the plasmids were no longer required, as the genes will be integrated into SynXIV by their loxP sites. To address these issues, the SCRaMbLEd strains were grown on a minimal medium similar to the recombinant strains, but with all amino acids added (section 2.1 Materials and Methods) to ensure the previously reported fatty acid amounts before and after SCRaMbLE were comparable. Additionally, this will ensure any changes in fatty acids are not a result of the plasmids but rather the genes which are incorporated into the genome.

The fatty acid profiles of the SCRaMbLEd strains Syn-sXIV, Syn-s*ACC1* and Syn-s*FAS2* cultured in biological triplicates were then compared to the non-scrambled Syn-XIV, Syn-*ACC1* and Syn-*FAS2* (Figure 21). Surprisingly, there was a slight increase in fatty acid content in the Syn-sXIV strain. This could be a result of "self- SCRaMbLE" of the synthetic chromosome generating an improved gene expression leading to enhanced fatty acids and

lipid production. This could also simply be a result of the nature of the screening process, which targeted better producing fatty acid strains from the general SynXIV population.

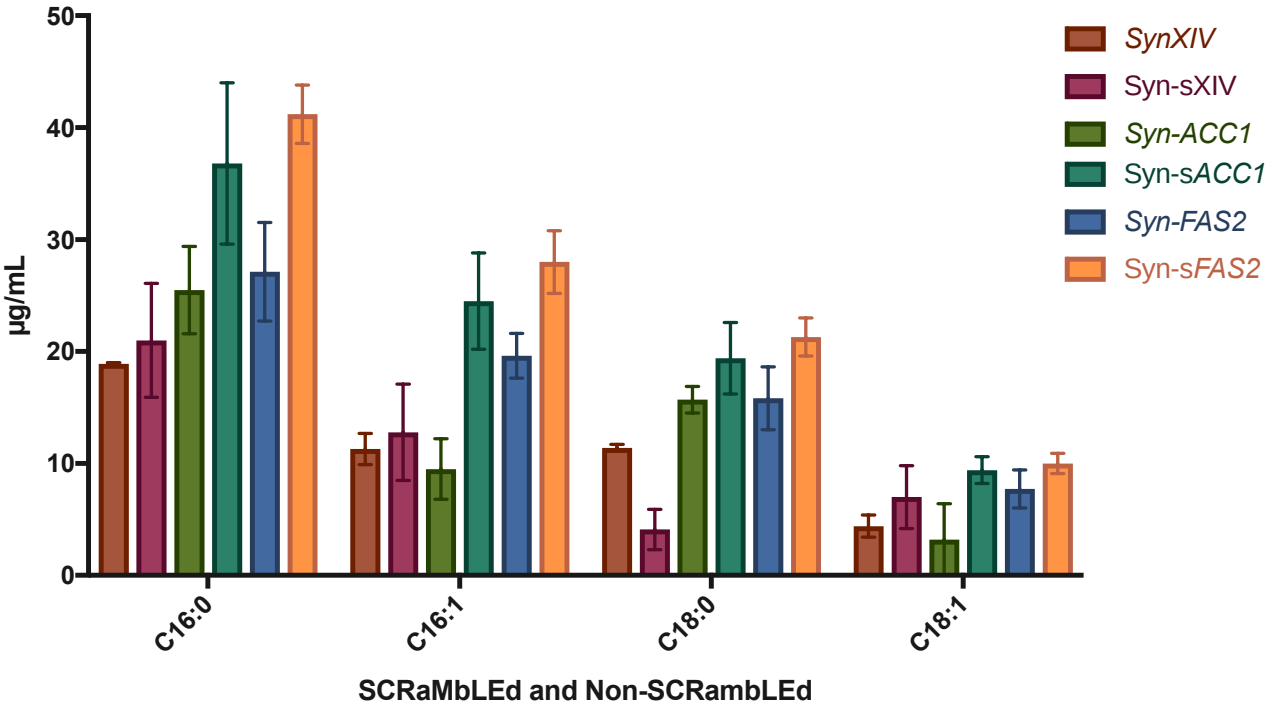

***Fig 21**. A comparison of the non-SCRaMbLEd strains and the SCRaMbLEd strains. Shown by SynXIV and Syn-sXIV there is a slight increase in C16:0, C16:1 and C18:1 in the Syn-sXIV strain with a decrease in C18:0. For Syn-ACC1 and Syn-sACC1 there is a considerable increase in all the C16 to C18 fatty acids with the greatest increases shown by C16:1 and C18:1. For Syn-FAS2 and Syn-sFAS2 there is an overall increase in all fatty acids C16 to C18. Syn-sFAS2 also shows the greatest accumulation of fatty acids when compared to all other strains. These results suggest SCRaMbLE is capable of increasing fatty acids levels even when a single gene is used.*

Both Syn-s*ACC1* and Syn-s*FAS2* showed an increase in their fatty acid content when compared to their non-SCRaMbLEd parent strains (Fig. 21). This is also shown by the combined total of C16:0, C16:1, C18:0 and C18:1 FAs in Fig. 22. Interestingly, by SCRaMbLEing *ACC1* the resulting new strain was capable of making 52% more fatty acids when compared to the recombinant strain and 60% more fatty acids than the original SynXIV strain. While, Syn-s*FAS2,* was able to produce 36% more fatty acids than Syn-*FAS2* and 80% more fatty acids than SynXIV. Syn-s*FAS2* has the greatest fatty acid titre improvement. This could be due to its strong general involvement in the fatty acid elongation process from Acyl-CoA (C3:0) to steric acid (C18:0) (Fig. 1 Introduction). Although, without sequencing analysis only assumptions can be made.

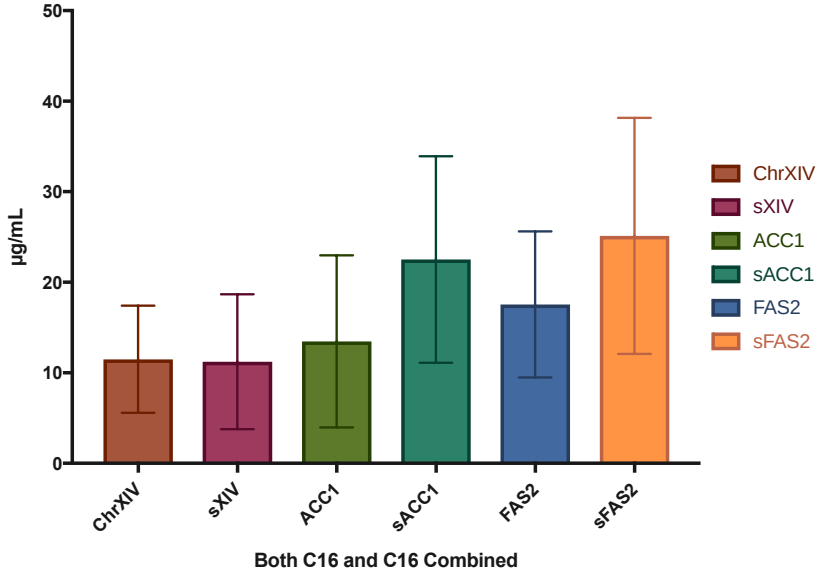

***Fig. 22**. The three SCRaMbLEd strains compared to the original non-SCRaMbLEd strains. The total fatty acid (C16:0, C16:1, C18:0 and C18:1) is compiled here. There is no comparable difference between the Syn-XIV strain and Syn-sXIV while Syn-sACC1 has double the total amounts of fatty acids than Syn-ACC1 and Syn-sFAS1 has almost double the amount of Syn-FAS2. Note, large error bars are a result of the combined errors between C16:0, C16:1, C18:0 and C18:1 result.*

On the other hand, *ACC1* is heavily regulated as it is one of the limiting factors in the fatty acid synthesis pathway [49]. Surprisingly, a single gene SCRaMbLEd into the synthetic chromosome was able to shift the carbon flux within the cell to produce more fatty acids than required by the cell. This demonstrates the combined power of SCRaMbLE technology and the 'purpose-built' screening platform developed in this work to create strains capable of redirecting carbon flux towards desired products fatty acid products.

Chapter 4 Conclusion

Overall, the project achieved its main goal, which was to build a platform for enhancing the production of fatty acids in *S. cerevisiae*. A specific, robust and workable high throughput screening protocol using flow cytometry, developed in this work was shown to distinguish high fatty acid producing cells from a heterologous mixture of recombinant yeasts. SCRaMbLE performed with the recombinant strains produced new strains with considerably higher fatty acid levels. This was positive, although somewhat unexpected especially considering only a single gene type was SCRaMbLEd in. Also shown in this work was assumed that a killing assay could be used to measure efficacy of the SCRaMbLE or merely to confirm SCRaMbLE occurred. Although, this is suspected without performing appropriate sequencing analysis and therefore cannot be confirmed.

After SCRaMbLE, Syn-s*ACC1* produced 60% more fatty acids than the SynXIV strain and Syn-s*FAS2* produced 80% more fatty acids than the SynXIV. These results support further the notion that even a single gene SCRaMbLE can be very useful in providing desired products. Sequencing of the genomes of the SCRaMbLEd Syn-s*ACC1* and Syn-s*FAS2* will provide detailed information of the specific genotypic changes resulting in the increased production of fatty acids. It would also be beneficial to revisit the screening protocol to work out reasons for the low number of SCRaMbLEd survivors coming through to increase the number of strains to be analysed further.

The *FAS1* and *FAS2* genes function together forming the FAS multienzyme complex, due to this it can only be assumed that by overexpressing only one of the two genes at a time the complex's efficiency will be hindered. In future work, to take advantage of the whole fatty acid elongation process in yeast, *FAS1* and *FAS2* must be co-SCRaMbLEd to ensure an efficient pathway, which would allow for the maximum number for fatty acids to be synthesised. Another problem not addressed in this work are the regulatory enzymes and transcription factors involved in the fatty acid biosynthesis pathway, which function to prevent fatty acid overexpression. To overcome regulatory factors in the pathway, several genes would need to be deleted or at least repressed to allow any strain to exceed the theoretical limits of fatty acid production in yeast. Such genes include *FAA1-4*, *POX 1-3*, *FOX2* and *3* (Fig. 2). Acknowledging there are over a dozen other regulatory factors, these genes would be an attainable starting point. To that end, without uncoupling the yeast fatty acid synthesis regulatory network currently a SCRaMbLEd strain would be unlikely be able to compete with the palm oil industry to provide an alternative source.

Another goal is to improve the screening protocol. As one of the desired products, oleic acid, plays a regulatory role in the controlling fatty acid synthesis. This could be examined further to generate a feedback biosensor within a plasmid expressing GFP in the presence of oleic acid, thus providing another (additional) way to gate populations to further improve the screening power.

Nevertheless, the platform developed in this work, in which SCRaMbLE technology can be exploited to improve production of the fatty acids of interest, will be applicable for the future development of fatty acid production in yeast. Therefore, a palm oil producing yeast seems an achievable target.

References:

1. Carter, C., et al., *Palm oil markets and future supply.* European Journal of Lipid Science and Technology, 2007. 109(4): p. 307-314.
2. Che Man, Y., et al., *Composition and thermal profile of crude palm oil and its products.* Journal of the American Oil chemists' Society, 1999. 76(2): p. 237-242.
3. Prokurat, S., *Palm oil–strategic source of renewable energy in Indonesia and Malaysia.* 2013.
4. Fitzherbert, E.B., et al., *How will oil palm expansion affect biodiversity?* Trends in Ecology & Evolution, 2008. 23(10): p. 538-545.
5. Lamers, D., et al., *Selection of oleaginous yeasts for fatty acid production.* BMC Biotechnology, 2016. 16(1): p. 45.
6. Tai, M. and G. Stephanopoulos, *Engineering the push and pull of lipid biosynthesis in oleaginous yeast Yarrowia lipolytica for biofuel production.* Metabolic Engineering, 2013. 15: p. 1-9.
7. Baker, T.A., et al., *Molecular biology of the gene*. 2003: Benjamin-Cummings Publishing Company.
8. Bücking-Throm, E., et al., *Reversible arrest of haploid yeast cells at the initiation of DNA synthesis by a diffusible sex factor.* Experimental Cell Research, 1973. 76(1): p. 99-110.
9. Gietz, R.D. and R.A. Woods, *Yeast transformation by the LiAc/SS Carrier DNA/PEG method*, in *Yeast Protocol*. 2006, Springer. p. 107-120.
10. Mewes, H., et al., *Overview of the yeast genome.* Nature, 1997. 387(6632): p. 7-8.
11. Sheng, J. and X. Feng, *Metabolic engineering of yeast to produce fatty acid-derived biofuels: bottlenecks and solutions.* Frontiers in Microbiology, 2015. 6: p. 554.
12. Runguphan, W. and J.D. Keasling, *Metabolic engineering of Saccharomyces cerevisiae for production of fatty acid-derived biofuels and chemicals.* Metabolic Engineering, 2014. 21: p. 103-113.
13. Tang, X., H. Feng, and W.N. Chen, *Metabolic engineering for enhanced fatty acids synthesis in Saccharomyces cerevisiae.* Metabolic Engineering, 2013. 16: p. 95-102.
14. Leber, C., et al., *Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in Saccharomyces cerevisiae.* Metabolic Engineering, 2015. 28: p. 54-62.
15. Zhang, Y., J. Nielsen, and Z. Liu, *Metabolic engineering of Saccharomyces cerevisiae for production of fatty acid-derived hydrocarbons.* Biotechnology and Bioengineering, 2018.
16. Valle-Rodríguez, J.O., et al., *Metabolic engineering of Saccharomyces cerevisiae for production of fatty acid ethyl esters, an advanced biofuel, by eliminating non-essential fatty acid utilization pathways.* Applied Energy, 2014. 115: p. 226-232.
17. Zhou, Y.J., et al., *Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories.* Nature Communications, 2016. 7: p. ncomms11709.
18. Ferreira, R., et al., *Metabolic engineering of Saccharomyces cerevisiae for overproduction of triacylglycerols.* Metabolic Engineering Communications, 2018. 6: p. 22-27.
19. Leber, C., et al., *Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in Saccharomyces cerevisiae.* Metabolic Engineering, 2015. 28: p. 54-62.
20. Czabany, T., K. Athenstaedt, and G. Daum, *Synthesis, storage and degradation of neutral lipids in yeast.* Biochimica et Biophysical Acta (BBA)-Molecular and Cell Biology of Lipids, 2007. 1771(3): p. 299-309.
21. Kohlwein, S.D., *Triacylglycerol homeostasis: insights from yeast.* Journal of Biological Chemistry, 2010: p. jbc. R110. 118356.
22. Roggenkamp, R., S. Numa, and E. Schweizer, *Fatty acid-requiring mutant of Saccharomyces cerevisiae defective in acetyl-CoA carboxylase.* Proceedings of the National Academy of Sciences, 1980. 77(4): p. 1814-1817.
23. Thevelein, J.M. and S. Hohmann, *Trehalose synthase: guard to the gate of glycolysis in yeast?* Trends in Biochemical Sciences, 1995. 20(1): p. 3-10.

24. Fleischer, T.C., et al., *Systematic identification and functional screens of uncharacterized proteins associated with eukaryotic ribosomal complexes.* Genes Deviation, 2006. 20(10): p. 1294-307.
25. Yang, H., et al., *Sterol esterification in yeast: a two-gene process.* Science, 1996. 272(5266): p. 1353-6.
26. Chae, M., G.S. Han, and G.M. Carman, *The Saccharomyces cerevisiae actin patch protein App1p is a phosphatidate phosphatase enzyme.* Journal of Biological Chemistry, 2012. 287(48): p. 40186-96.
27. Calder, R.B., et al., *Cloning and characterization of a eukaryotic pantothenate kinase gene (panK) from Aspergillus nidulans.* Journal of Biological Chemistry, 1999. 274(4): p. 2014-20.
28. Oelkers, P., et al., *The DGA1 gene determines a second triglyceride synthetic pathway in yeast.* Journal of Biological Chemistry, 2002. 277(11): p. 8877-8881.
29. Gajdos, P., et al., *Single cell oil production on molasses by Yarrowia lipolytica strains overexpressing DGA2 in multicopy.* Appl Microbiol Biotechnol, 2015. 99(19): p. 8065-74.
30. Han, G.S., et al., *Characterization of the yeast DGK1-encoded CTP-dependent diacylglycerol kinase.* Journal of Biological Chemistry, 2008. 283(29): p. 20443-53.
31. Toke, D.A., et al., *Isolation and characterization of the Saccharomyces cerevisiae DPP1 gene encoding diacylglycerol pyrophosphate phosphatase.* Journal of Biological Chemistry 1998. 273(6): p. 3278-84.
32. Duronio, R.J., L.J. Knoll, and J.I. Gordon, *Isolation of a Saccharomyces cerevisiae long chain fatty acyl:CoA synthetase gene (FAA1) and assessment of its role in protein N-myristoylation.* Journal of Cell Biology, 1992. 117(3): p. 515-29.
33. Knoll, L.J., D.R. Johnson, and J.I. Gordon, *Biochemical studies of three Saccharomyces cerevisiae acyl-CoA synthetases, Faa1p, Faa2p, and Faa3p.* Journal of Biological Chemistry, 1994. 269(23): p. 16348-56.
34. Johnson, D.R., et al., *Saccharomyces cerevisiae contains four fatty acid activation (FAA) genes: an assessment of their role in regulating protein N-myristoylation and cellular lipid metabolism.* Journal of Cell Biology, 1994. 127(3): p. 751-62.
35. Schweizer, E. and J. Hofmann, *Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems.* Microbiology and Molecular Biology Reviews, 2004. 68(3): p. 501-517.
36. Mortimer, R.K., et al., *Genetic map of Saccharomyces cerevisiae, edition 10.* Yeast, 1989. 5(5): p. 321-403.
37. Hiltunen, J.K., et al., *The biochemistry of peroxisomal beta-oxidation in the yeast Saccharomyces cerevisiae.* FEMS Microbiology Reviews, 2003. 27(1): p. 35-64.
38. Ayciriex, S., et al., *YPR139c/LOA1 encodes a novel lysophosphatidic acid acyltransferase associated with lipid droplets and involved in TAG homeostasis.* Molecular Biology Cell, 2012. 23(2): p. 233-46.
39. Toke, D.A., et al., *Isolation and characterization of the Saccharomyces cerevisiae LPP1 gene encoding a Mg2+-independent phosphatidate phosphatase.* Journal of Biological Chemistry, 1998. 273(23): p. 14331-8.
40. Hiltunen, J.K., et al., *The biochemistry of peroxisomal β-oxidation in the yeast Saccharomyces cerevisiae.* FEMS Microbiology Reviews, 2003. 27(1): p. 35-64.
41. Adeyo, O., et al., *The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets.* The Journal of Cell Biology, 2011. 192(6): p. 1043-1055.
42. Erdmann, R., *The peroxisomal targeting signal of 3-oxoacyl-CoA thiolase from Saccharomyces cerevisiae.* Yeast, 1994. 10(7): p. 935-44.
43. Dmochowska, A., et al., *Structure and transcriptional control of the Saccharomyces cerevisiae POX1 gene encoding acyl-coenzyme A oxidase.* Gene, 1990. 88(2): p. 247-52.

44. Athenstaedt, K. and G. Daum, *Biosynthesis of phosphatidic acid in lipid particles and endoplasmic reticulum of Saccharomyces cerevisiae.* Journal of Bacteriology, 1997. 179(24): p. 7611-6.
45. Koffel, R., et al., *The Saccharomyces cerevisiae YLL012/YEH1, YLR020/YEH2, and TGL1 genes encode a novel family of membrane-anchored lipases that are required for steryl ester hydrolysis.* Molcular Cell Biology, 2005. 25(5): p. 1655-68.
46. Athenstaedt, K. and G. Daum, *Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast Saccharomyces cerevisiae are localized to lipid particles.* Journal of Biological Chemistry, 2005. 280(45): p. 37301-9.
47. Stukey, J.E., V.M. McDonough, and C.E. Martin, *The OLE1 gene of Saccharomyces cerevisiae encodes the delta 9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene.* Journal of Biological Chemistry, 1990. 265(33): p. 20144-9.
48. Van den Berg, M.A., et al., *The two acetyl-coenzyme A synthetases of Saccharomyces cerevisiae differ with respect to kinetic properties and transcriptional regulation.* Journal of Biological Chemistry, 1996. 271(46): p. 28953-9.
49. Hasslacher, M., et al., *Acetyl-CoA carboxylase from yeast is an essential enzyme and is regulated by factors that control phospholipid metabolism.* Journal of Biological Chemistry, 1993. 268(15): p. 10946-52.
50. Kolodziej, S.J., et al., *Structure-function relationships of the Saccharomyces cerevisiae fatty acid synthase. Three-dimensional structure.* Journal of Biological Chemistry, 1996. 271(45): p. 28422-9.
51. Tang, X., J. Lee, and W.N. Chen, *Engineering the fatty acid metabolic pathway in Saccharomyces cerevisiae for advanced biofuel production.* Metabolic Engineering Communications, 2015. 2: p. 58-66.
52. Schneiter, R. and S.D. Kohlwein, *Organelle structure, function, and inheritance in yeast: a role for fatty acid synthesis?* Cell, 1997. 88(4): p. 431-434.
53. Tehlivets, O., K. Scheuringer, and S.D. Kohlwein, *Fatty acid synthesis and elongation in yeast.* Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 2007. 1771(3): p. 255-270.
54. McDonough, V.M., J.E. Stukey, and C.E. Martin, *Specificity of unsaturated fatty acid-regulated expression of the Saccharomyces cerevisiae OLE1 gene.* Journal of Biological Chemistry, 1992. 267(9): p. 5931-6.
55. Rottensteiner, H., et al., *Pip2p: a transcriptional regulator of peroxisome proliferation in the yeast Saccharomyces cerevisiae.* Embo journal, 1996. 15(12): p. 2924-34.
56. Wang, Q., et al., *A critical role for very long-chain fatty acid elongases in oleic acid-mediated Saccharomyces cerevisiae cytotoxicity.* Microbiology Reviews, 2018. 207: p. 1-7.
57. Simon, I., et al., *Serial regulation of transcriptional regulators in the yeast cell cycle.* Cell, 2001. 106(6): p. 697-708.
58. Leber, R., et al., *Characterization of lipid particles of the yeast, Saccharomyces cerevisiae.* Yeast, 1994. 10(11): p. 1421-8.
59. Kohlwein, S.D., *Triacylglycerol homeostasis: insights from yeast.* Journal of Biological Chemistry, 2010. 285(21): p. 15663-7.
60. Athenstaedt, K., et al., *Identification and characterization of major lipid particle proteins of the yeast Saccharomyces cerevisiae.* Journal Bacteriology, 1999. 181(20): p. 6441-8.
61. Huang, N.L., et al., *Oleosin of subcellular lipid droplets evolved in green algae.* Plant Physiology, 2013. 161(4): p. 1862-74.
62. Hsieh, K. and A.H. Huang, *Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells.* Plant Physiology, 2004. 136(3): p. 3427-34.
63. Brasaemle, D.L., *Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis.* Journal of Lipid Reviews, 2007. 48(12): p. 2547-59.

64. Napier, J.A., A.K. Stobart, and P.R. Shewry, *The structure and biogenesis of plant oil bodies: the role of the ER membrane and the oleosin class of proteins.* Plant Mololcular Biology, 1996. 31(5): p. 945-56.
65. Radulovic, M., et al., *The emergence of lipid droplets in yeast: current status and experimental approaches.* Current Genetics, 2013. 59(4): p. 231-42.
66. Athenstaedt, K. and G. Daum, *The life cycle of neutral lipids: synthesis, storage and degradation.* Cell Molecular Life Sciences, 2006. 63(12): p. 1355-69.
67. Rajakumari, S., K. Grillitsch, and G. Daum, *Synthesis and turnover of non-polar lipids in yeast.* Programable Lipid Reviews, 2008. 47(3): p. 157-71.
68. Robenek, H., et al., *Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis.* Journal of Cell Science, 2006. 119(Pt 20): p. 4215-24.
69. Athenstaedt, K., et al., *Lipid particle composition of the yeast Yarrowia lipolytica depends on the carbon source.* Proteomics, 2006. 6(5): p. 1450-9.
70. Erdmann, R., et al., *Isolation of peroxisome-deficient mutants of Saccharomyces cerevisiae.* Proceddings of the National Acdemy of Sciences America, 1989. 86(14): p. 5419-23.
71. Rosenberger, S., et al., *Phosphatidylethanolamine synthesized by three different pathways is supplied to peroxisomes of the yeast Saccharomyces cerevisiae.* Biochimica et Biophysica Acta, 2009. 1791(5): p. 379-87.
72. Connerth, M., et al., *Oleate inhibits steryl ester synthesis and causes liposensitivity in yeast.* Journal of Biological Chemistry, 2010. 285(35): p. 26832-41.
73. Andrianantoandro, E., et al., *Synthetic biology: new engineering rules for an emerging discipline.* Molcular Systems Biology, 2006. 2: p. 2006.0028.
74. James, J.R. and R.D. Vale, *Biophysical mechanism of T-cell receptor triggering in a reconstituted system.* Nature, 2012. 487(7405): p. 64-9.
75. Dormitzer, P.R., et al., *Synthetic generation of influenza vaccine viruses for rapid response to pandemics.* Science Translational Medicine, 2013. 5(185): p. 185ra68.
76. Richardson, S.M., et al., *Design of a synthetic yeast genome.* Science, 2017. 355(6329): p. 1040-1044.
77. Shen, Y., et al., *SCRaMbLE generates designed combinatorial stochastic diversity in synthetic chromosomes.* Genome Resolution, 2016. 26(1): p. 36-49.
78. Blount, B.A., et al., *Rapid host strain improvement by in vivo rearrangement of a synthetic yeast chromosome.* Nature Communications, 2018. 9(1): p. 1932.
79. Shen, M.J., et al., *Heterozygous diploid and interspecies SCRaMbLEing.* Nature Communications, 2018. 9(1): p. 1934.
80. Jia, B., et al., *Precise control of SCRaMbLE in synthetic haploid and diploid yeast.* Nat Commun, 2018. 9(1): p. 1933.
81. Luo, Z., et al., *Identifying and characterizing SCRaMbLEd synthetic yeast using ReSCuES.* Nature Communications, 2018. 9(1): p. 1930.
82. Wu, Y., et al., *In vitro DNA SCRaMbLE.* Nature Communications, 2018. 9(1): p. 1935.
83. Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular cloning: a laboratory manual*. 1989: Cold Spring Harbor Laboratory Press.
84. Kearse, M., et al., *Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data.* Bioinformatics, 2012. 28(12): p. 1647-1649.
85. Sikorski, R.S.H., Philip, *A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae.* Genetics, 1989. 122(1): p. 19-27.
86. Annaluru, N., et al., *Total synthesis of a functional designer eukaryotic chromosome.* Science, 2014. 344(6179): p. 55-58.
87. Lõoke, M., K. Kristjuhan, and A. Kristjuhan, *Extraction of genomic DNA from yeasts for PCR-based applications.* Biotechniques, 2011. 50(5): p. 325.

88. Froger, A. and J.E. Hall, *Transformation of plasmid DNA into E. coli using the heat shock method.* Journal of Visualized Experiments: JoVE, 2007(6).
89. Pellicciari, C., M. Biggiogera, and Pellicciari, *Histochemistry of Single Molecules*. 2017: Springer.
90. Bligh, E.G. and W.J. Dyer, *A rapid method of total lipid extraction and purification.* Canadian Journal of Biochemistry and Physiology, 1959. 37(8): p. 911-917.
91. Richardson, S.M., et al., *Design of a synthetic yeast genome.* Science, 2017. 355(6329): p. 1040-1044.
92. Siloto, R.M., et al., *Simple methods to detect triacylglycerol biosynthesis in a yeast-based recombinant system.* Lipids, 2009. 44(10): p. 963.
93. Werner-Washburne, M., et al., *Stationary phase in the yeast Saccharomyces cerevisiae.* Microbiological Reviews, 1993. 57(2): p. 383-401.
94. De Deken, R., *The Crabtree effect: a regulatory system in yeast.* Microbiology, 1966. 44(2): p. 149-156.
95. Rattray, J., A. Schibeci, and D.K. Kidby, *Lipids of yeasts.* Bacteriological Reviews, 1975. 39(3): p. 197.
96. Jovicevic, D., B.A. Blount, and T. Ellis, *Total synthesis of a eukaryotic chromosome: Redesigning and SCRaMbLE-ing yeast.* Bioessays, 2014. 36(9): p. 855-60.