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Optimization of transfection of primary hepatocytes from Atlantic salmon for functional studies

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Acknowledgments

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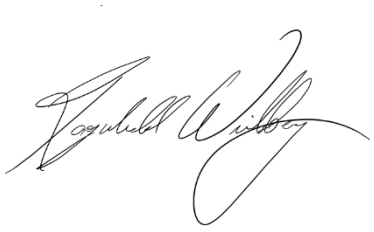

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Abstract

Gene expression in eukaryotes are regulated through complex interactions between regulatory proteins, cis-regulatory sequences as well as chemical modifications and structure of chromatin. The function of cis-regulatory regions can be studied through expression of reporter genes in cell cultures. Ideally such reporter gene expression should be studied in biologically relevant cells, like primary cell cultures. The primary aim of this work was to optimize a protocol for transfection of primary hepatocytes from Atlantic salmon, to be able to perform functional studies on gene expression in liver. The secondary aim was to test this protocol by performing a promoter reporter assay on the cis-regulatory elements driving the expression in two duplicated Atlantic salmon fatty acyl elongase 5 genes involved in the endogenous synthesis of long-chain polyunsaturated fatty acid (LC-PUFA) synthesis in liver.

Transfection optimizations were done using both cationic lipid-mediated transfection and electroporation. Results from these optimization experiments suggests that electroporation is the superior option for transfecting primary Atlantic salmon hepatocytes, with transfection efficiency up to 30%. We also found that perfusion is an important, but technically challenging element for successful transfection in these cells. Finally, our experiment with the *elovl5* promoter sequences showed induced reporter expression in hepatocytes.

In conclusion, we have developed a robust transfection protocol which paving the way for functional studies of gene regulatory logics in liver cells.

Sammendrag

Genuttrykk i eukaryoter er regulert gjennom komplekse interaksjoner mellom regulatoriske proteiner, cis-regulatoriske sekvenser og kjemiske og strukturelle endringer av kromatin. Funksjonen av cis-regulatoriske sekvenser kan studeres gjennom uttrykk av reportergener i cellekulturer. Ideelt sett bør slike reportergenstudier utføres i biologisk relevante celler, som for eksempel primæcellekulturer. Hovedmålet med dette arbeidet var derfor å optimalisere transfeksjon av primære hepatocytter fra atlanterhavslaks, for å kunne utføre funksjonelle studier av genuttrykk i lever. Det sekundære målet var å teste denne protokollen ved å sammenlikne cis-regulatoriske sekvenser fra to *elovl5*-genduplikater i atlanterhavslaks. Disse to genene koder for elovl5-enzymer som er involvert i den endogene syntesen av langkjedede flerumettede fettsyrer i lever.

Transfeksjonsoptimaliseringen ble gjort både for kationisk lipid-mediert tranfeksjon og elektroporering. Resultatene fra optimaliseringsforsøkene viste at elektroporering er det mest effektive alternativet for å transfektere primære hepatocytter fra atlanterhavslaks, med en transfeksjonseffiktivitet på 30%. I tillegg fant vi at perfusjon er et viktig, men teknisk utfordrende element for vellykket transfeksjon. Til sist viste vi at elovl5-promotere kan indusere transkripsjon i primære leverceller.

Vi konkluderer med at vår nye transfeksjonsprotokoll vil kunne være et viktig verktøy for fremtidige studier av genregulering i leverceller.

Contents

1 Introduction

1.1 Transcriptional regulation

Gene regulation encompass a range of processes which together control the production of gene products (RNA or protein), and ultimately gives each cell and tissue their unique function. These gene regulatory processes are often subdivided into two main levels, transcriptional and post transcriptional regulation. The former is the regulation of transcription of DNA to RNA, while the latter is all processes that can modify the production of protein from those RNA molecules (**Figure 1**). In this thesis our main aim is is to optimize an *in vitro* system for studying transcriptional regulation and in the following sections I will therefore briefly outline the principles of gene transcription and transcriptional regulation.

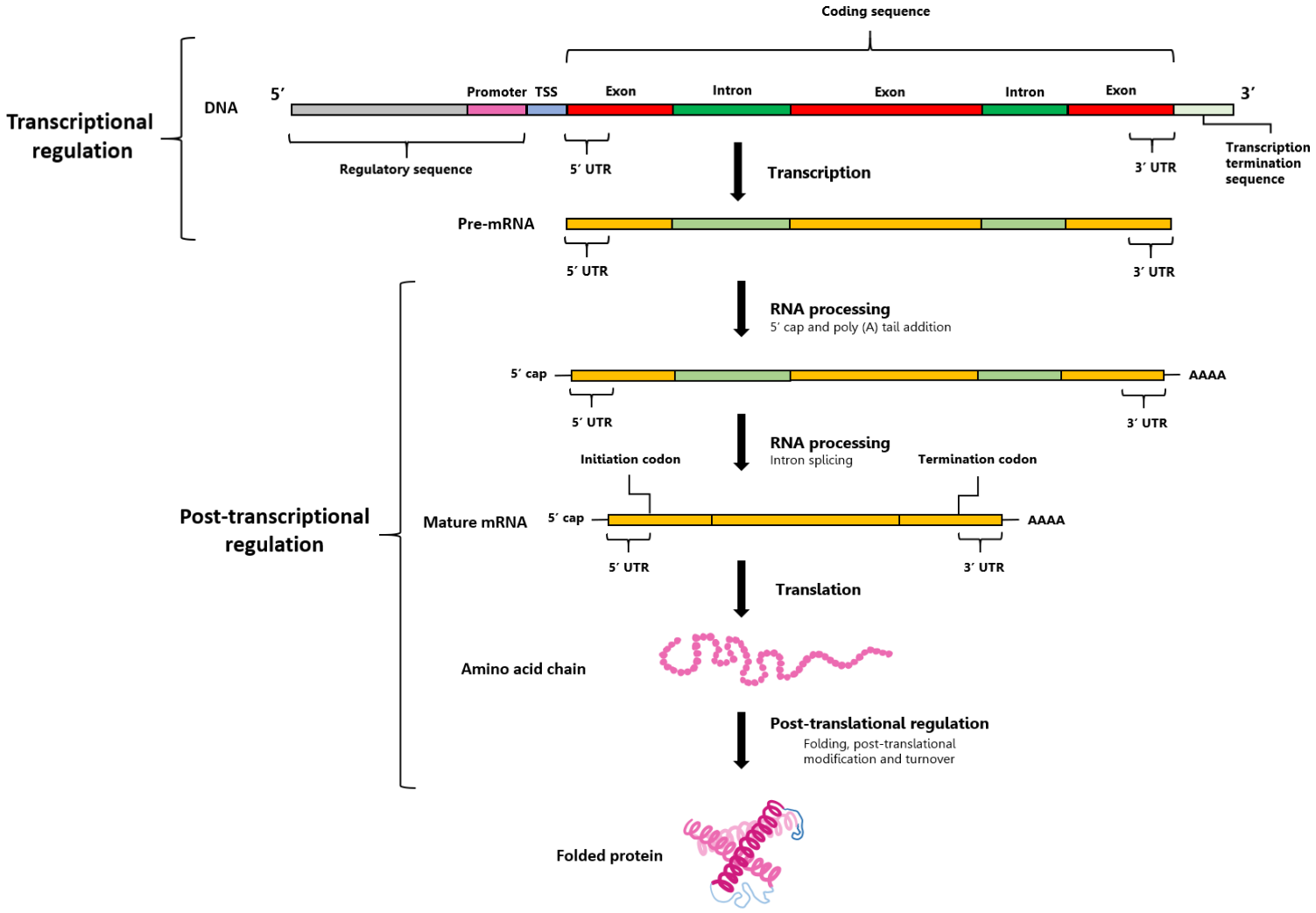


Figure 1. The levels of gene regulation. DNA is transcribed to pre-mRNA. After transcription, the pre-mRNA is added a 5′ cap and a poly (A) tail at the 3′ end and introns are spliced out, enabling alternative splicing. Mature mRNA moves out to the cytoplasm where translation and further post-translational modification occurs.

Transcription depend on RNA polymerase being recruited to the DNA. For that to happen general transcription factors (GTFs) needs to bind the core promoter. The core promoter is usually defined as the ±50 bp flanking the transcription start site (TSS) (Andersson & Sandelin, 2020; Haberle & Stark, 2018). The core promoter, together with bound GTFs and RNA polymerase form the basal transcriptional machinery (Field & Adelman, 2020). In addition to the general transcription factors, additional transcription factors (TFs) further affect transcription rate by influencing the RNA polymerase recruitment, initiation and elongation (Andersson & Sandelin, 2020). TFs are regulatory proteins, that recognize and bind short specific DNA sequences (motifs) located both upstream and occasionally downstream of the core promoter, referred to as cis-regulatory elements (CREs). These CREs can be either promoters or enhancers. Both terms refer to DNA sequences that bind regulatory proteins (TFs) that regulate the activity of the RNA polymerase. The promoter sequence is usually situated upstream of the TSS and includes the core promoter. Pinpointing the promoter region is often challenging and requires promoter bashing, which involves introducing targeted point mutations or deletions in the promoter sequence and subsequent measurment of transcriptional activity (Andersson & Sandelin, 2020). Compared to promoters, enhancers are independent of direction, and positioned further away from the core promoter (Field & Adelman, 2020). This distance can stretch up to 1 Mb (Andersson & Sandelin, 2020). Traditionally there has been made a clear distinction between promoter and enhancer elements, but more recent studies challenge this traditional dichotomy and rather emphasizes the similarities between promoters and enhancers. It has been suggested that it might be more appropriate to classisfy CRE's as regulatory elements that can have both promoter and enhancer potential (Andersson, 2015; Andersson & Sandelin, 2020; Field & Adelman, 2020).

Transcription factors bound away from the core promoter act indirectly on the RNA polymerase through the mediator complex (Field & Adelman, 2020). Spatial proximity to this complex is achieved by folding of the DNA (Soutourina, 2017). Cooperative binding of TFs to the DNA is common and occur in so called cis-regulatory modules (Andersson & Sandelin, 2020). Other proteins, like co-activators and co-repressors are also involved in this highly dynamic and competitive complex formation. Promoter strength describes how effectively a promoter can recruit the polymerase and how tightly the RNA polymerase binds to the DNA and depends on the nucleotide sequence of the cis-regulatory regions of the gene (Kiryu et al., 2005). The promoter strength affects the transcription efficiency and therby the gene expression level.

Transcription factor binding to CREs relies on DNA accessibility (Klemm et al., 2019). This implies that there is a histone depletion at the regulatory loci of actively transcribed genes (Klemm et al., 2019; Lee et al., 2004). The chromatin accessibility is itself modulated by so called histone modifications, like methylations, phosphorylations and acetylations. As such, the transcription regulation is in the end a combinatorial function of the the interplay between cellular regulation of TF proteins, cis-regulatory elements, and the chromatin accessibility.

1.2 Promoter reporter assays

A method used to gain mechanistic insights into how transcription of a target gene is regulated through cis-regulatory elements (TFBS) is promoter reporter assays (PRA). In brief, these methods rely on two experimental steps (**Figure 2**): Firstly, cells must be transfected with a promoter reporter construct containing CREs of interest. Secondly, the impact of the CREs on the transcription of the reporter gene needs to be quantified. These methods enable identification of cis-regulatory elements that influences transcription of the target gene. There are many different types of PRAs methods, varying both in the method to deliver reporter constructs to the cells of interest (e.g. in vitro cell cultures or in vivo delivery in developing embryos) and quantification of transcription output.

In this thesis we have focused on optimizing a luciferase reporter assay (LUC-PRA) in hepatic primary cell cultures of Atlantic salmon. For luciferase reporter assays cells are transfected with plasmid vectors containing the cis-regulatory region of interest followed by a Firefly Luciferase reporter gene, followed by quantification of Luciferase activity. The expression level of the Luciferase protein relies on transcription factors binding to the CREs and thereby stimulating transcriptional activity. The resulting bioluminescent signal should correspond to level of gene expression. Experimental conditions can cause sample variations such as differences in transfection efficiency, viability and cell number (Branchini et al., 2018). For this reason, cells are co-transfected with a normalizing control vector, which in our approach was a renilla luciferase. The firefly signal and the renilla signal is measured sequentially due to their respective bioluminescent signal being a product of two different enzymatic reactions which require different substrates to be activated (Branchini et al., 2018).

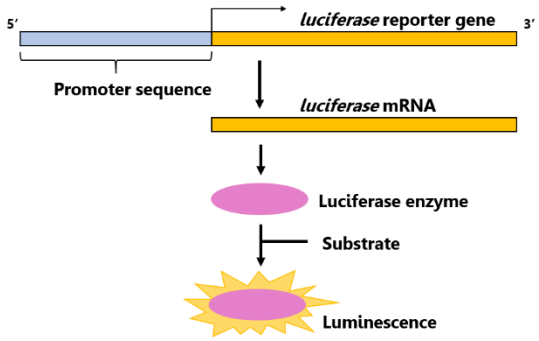


Figure 2. Schematic figure demonstrating the principle of luciferase assays. The translated Luciferase enzymes catalyze reactions that emits luminescence when reaction substrate is added.

1.3 Primary cell isolation

Transfection of many types of immortalized cell lines have become routine in todays labs, but transfection of primary cells is widely considered to be more challenging. Even so,

studying gene regulation in primary cells is considered more biologically relevant compared to cell lines (Gresch & Altrogge, 2012). Primary cell cultures retain many of their in vivo attributes for some time after isolation, making them more similar to the tissue in the living organism (Gresch & Altrogge, 2012). Immortalized cell lines often deviate from the native cell type in terms of which TFs that are expressed. In addition, long-term subculturing of cell lines may cause mutations and genetic drift which can alter the original characteristics of the cells. This could also be a source of unwanted variation (Uri et al., 2018).

To be able to transfect cells *in vitro* establishment of primary cell cultures is a prerequisite. However, there are several challenges with primary cell cultures, the two majors being viability after isolation and low or no proliferation. There are several ways of preparing primary culture, and one of these are by enzymatic digestion. A two-step perfusion technique for human hepatocytes was introduced in 1969 (Berry & Friend, 1969) and further improved in rat hepatocytes in 1976 (Seglen, 1976). This technique has since been widely applied to prepare primary hepatic cell cultures. The principle involves pumping enzymatic digestive buffer through the portal vein of the liver, thereby taking advantage of the venous system to reach the majority of the cells (Lee et al., 2013). The first perfusion is performed mostly to wash out blood cells. This buffer should however be free of Ca^{2+} or contain the metal ion chelating agent EDTA. Studies have found that Ca^{2+} removal facilitates the subseqeuent separation of cells by collagenase perfusion (Seglen, 1976). The effect of this initial Ca^{2+} removal is irreversible. For the subsequent collagenase perfusion, inclusion of Ca^{2+} is required for optimum collagenase activity. When perfusion is complete, the single cells are trapped inside the connective tissue and in the vascular extracellular matrix (Seglen, 1976). To release the cells, the tissue needs to be broken up with careful mechanical force, and gently shaken.

In addition to low cell viability, several other aspects of the primary cell culture negatively affect transfection efficiency, for example bacterial and/or fungal contamination and low cell density. These aspects are affected by the perfusion and the subsequent preparation of a single cell culture, but also general lab technique, choice of growth medium and potential additives like antibiotics and serum. The combinatorial effect of these elements does, in addition to the applied transfection technique, determine the transfection efficiency.

1.4 Transfection techniques

Achieving a high number of transfected cells is vital when performing promoter reporter assays. If only few cells express the reporter construct and the signal from the reporter gene will be difficult or impossible to measure. Two factors affect this: the number of transfected cells that survive (viability) but often more importantly, the proportion of cells that are transfected, i.e. the transfection efficiency. This greatly depends on efficient gene transfer. The choice of gene transfer technique depends on purpose and cell type. Ideally the transfection technique should give high transfection efficiencies, low cell toxicity and have minimal effects on normal cell physiology (Kim & Eberwine, 2010). For practical purposes, the method should also be easy to use and yield reproducible results.

Commonly, we distinguish between chemical and physical transfection techniques (**Figure 3 A-B**). All these methods involve foreign DNA being introduces in the cell and expressed, either transiently or stably. Transient transfection is applied for promoter reporter assays and entails the DNA being delivered into the nucleus without being integrated into the host genome (Kim & Eberwine, 2010). This causes the gene to only be expressed for a limited period after transfection. Stable transfection, however, involves the DNA being integrated into the host genome, ensuring expression after division.

There are several types of chemical transfection techniques. One of these is cationic lipid mediated transfection. Lipid mediated transfection is often viewed as a safe and relatively easy transfection teqhnique compared to other methods like virus-mediated transfection, which might be consideres more effective (Maurisse et al., 2010), but which entails exposure to biological hazards. Cationic lipid mediated transfection has also been shown to be effective in primary rat hepatocytes (Edwards et al., 2006; Gao et al., 2012; Park et al., 2011). This method involves mixing of negatively charged plasmids containing the reporter gene and spherical cationic lipid formations calles liposomes to create so called DNA:lipid lipoplexes with a positive surface charge (**Figure 3 A**). The DNA-containing complexes are presumably taken up by the cell either through the endocytic pathway or by membrane fusion. The efficiency of this method largely depends on the cell type, its division rate, its membrane composition and endocytic activity. A high cell division rate and endocytiv activity has long been thought to positively affects the transfection efficiency (Mortimer et al., 1999; Prasad et al., 2005; Riddle et al., 2007). which might pose a challenge when it comes to transfecting slow dividing cells like primary hepatocytes.

Some cell types are more resilient than others to foreign DNA uptake and might actively inhibit its endocytosis. The cell surface is covered with DNA pattern recognition receptors that can distinguish foreign DNA from the cells own DNA. These form part of the cells innate immune system. Such receptors also occupy the cytoplasmic space, and binding of foreign DNA can induce signal transduction cascade defense mechanisms (Bosnjak et al., 2018) and thereby inhibit transfection. Primary cells might potentially possess a more efficient innate immune system than cell lines due to exposure to selection pressure.

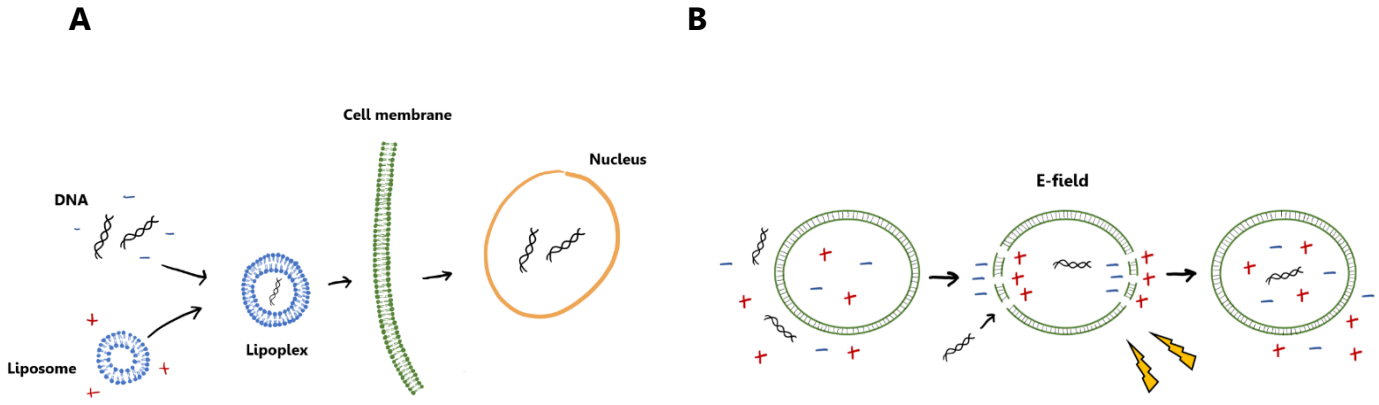


Figure 3. Transfection thechniques. A: Cationic lipid mediated transfection. DNA and liposomes are mixed to form positively charged lipoplexes. The DNA enters the cell either by the lipoplexes fusing with the cell membrane or by the endocytic pathway. **B:** Transfection by electroporation. It is hypothesized that applying an electric pulse to the cells generates an electric field across the cells that induces temporary pore formation through which the DNA can move and enter the cell and ultimately the nucleus.

The most used physical transfection technique is electroporation, which was first reported for gene transfer studies in mouse cells (Wong & Neumann, 1982). The method involves applying an electrical pulse that affects the phospholipids in the cell membrane so that they form transient pores, through which the nucleic acid can pass (Shigekawa & Dower, 1988) (**Figure 3 B**). In this case the cell membrane is practically forced open. This technique is less dependent on cell type than chemical transfection (Luft & Ketteler, 2015), and consequently bypasses many of the limitations of cationic lipid mediated transfection techniques. Electroporation is considered easy and fast, and has proven successful in hard-to-transfect cell types like primary cells (Jordan et al., 2008; Maurisse et al., 2010) Critical electroporation parameters include choice of electroporation buffer, voltage, pulse width and number of pulses applied. The primary disadvantage of electroporation is exposure to high voltage and incomplete healing of the cell membrane, both causing extensive cell damage and/or cell death. Some liposomal transfection reagents are also known to have cytotoxic effects. In addition, some activate stress response pathways that can give unintended changes in gene expression (Fiszer-Kierzkowska et al., 2011).

There are also many ways to quantify transfection success. Reporter systems like fluorescent proteins (FPs) can be used to monitor the fraction of transfected cells, enabling researchers to maximize transfection efficiency. Determining the fraction of FP expressing cells can be done by a combination of fluorescent microscopy and hemocytometry, or by flow cytometry. With flow cytometry the FP expression can be measured in a mixed population of cells based on the light scatter that is emitted when the cells are hit by a laser at the appropriate wavelength.

1.5 Proof of concept: CRE-divergence of Elovl5 duplicates

Although the main aim of this thesis was to develop efficient transfection protocols of primary hepatic cells, we also aimed to use this method to try to verify a previous published result on regulatory divergence between two gene duplicates (Carmona-Antoñanzas et al., 2016) as a proof of concept.

The Atlantic Salmon genome underwent a whole genome duplication event (WGD) ~80 million years ago (MYA) (Lien et al., 2016). Today, more than 50% of the genes in Atlantic salmon still have a retained duplicate originating from this WGD event, and about 50% of these retained duplicates have different tissue regulation (Lien et al. 2016). The evolutionary consequences of having such 'extra' gene copies have been extensively debated (Sandve et al., 2018). There are intriguing examples of salmonid specific gene duplicates which have evolved new potential adaptive gene regulation (**Figure 4**) and function (Carmona-Antoñanzas et al., 2016; Lien et al., 2016; Lorgen et al., 2015).

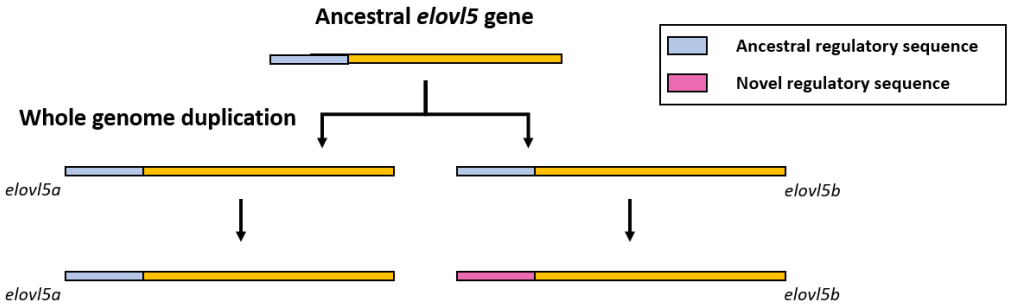


Figure 4. Diverging regulation in the *elovl5* duplicates. Schematic figure of diverging regulation in the *elovl5* duplicates after the Salmonid whole genome duplication ~80 mya.

One duplicate pair of Atlantic Salmon genes which have received attention in the scientific literature are the fatty acyl elongase 5 (Elovl5) genes, *elovl5a* and *elovl5b*. These genes belong to the elongase gene family that codes for enzymes involved with synthesis of long chain polyunsaturated fatty acids (LC-PUFAs) like docosahexenoic acid (DHA) and eicosapentenoic acid (EPA), by elongation of short chain polyunsaturated fatty acids (PUFAs) (Morais et al., 2009; Qin et al., 2009).

Unlike most vertebrates, Atlantic salmon are incapable of synthesizing LC-PUFA de novo (Leaver et al., 2014). This ability of to synthesize LC-PUFAs is particularly interesting in an evolutionary perspective. The most recent common ancestor of the Salmonids is presumed to be a pike-like fish (Varadharajan et al., 2018). Being piscivorous throughout it's lifespan, pikes have a diet rich in lipids, compared to the Salmon (Varadharajan et al., 2018). In early life, Salmon live in fresh water riverine habitat, with poor availability of dietary LC omega-3 fatty acids. As such, salmonids and other fresh water fish are hypothesized to have evolved a more efficient endogenous production of LC-PUFAs (Ishikawa et al., 2019; Ravia & Venkatesha, 2008; Varadharajan et al., 2018).

1.6 Transcriptional regulation of *elovl5a* and *elovl5b*

elovl5b are among highly expressed genes in hepatic cells in liver tissue in Atlantic salmon, but they are also expressed in other tissues, like the heart and gut (**Figure 4**). Although both *elovl5* duplicates are highly conserved at the protein sequence level (Carmona-Antoñanzas et al., 2016) the expression of *elovl5b* has been shown to be much higher than *elovl5a* in liver tissue (**Figure 4**). It's been suggested that this presumed regulatory neofunctionalization could increase flexibility in *elovl5* expression across tissues and under varying nutritional conditions (Carmona-Antoñanzas et al., 2016). The *elovl5* duplicates have been shown to exhibit divergent expression regulation in live Atlantic salmon as a results of changes in the lipid composition in their diet (Morais et al., 2009).

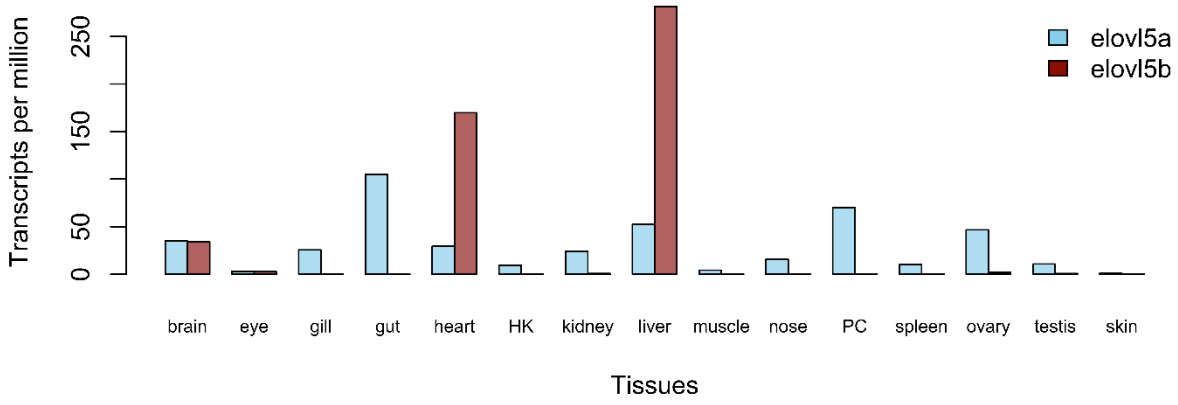


Figure 5. Tissue expression atlas for the *elovl5* duplicates in Atlantic salmon. Showing expression level differences between the *elovl5a* and *elovl5b* duplicates in different tissues of a single Atlantic salmon.

A recent study (Carmona-Antoñanzas et al., 2016) found that the duplicates may exhibit divergent expression levels because the regulatory regions of these genes have been asymmetrically colonised by transposable elements (TEs). The same study found that highly repeated elements accounts for 23% and 39% of the *elovl5b* and *elovl5a* promoter lengths, respectively. The TE insertions in the promoter regions of the *elovl5* duplicates likely appeared some time after the salmonid WGD (Carmona-Antoñanzas et al., 2016).
The evolution of gene regulation is known to be partly shaped by changes in the cis-regulatory sequence, by adding new, or disrupting already existing CREs (Mulugeta et al., 2019). Transposable elements have the potential to add such new cis-regulatory elements to already existing promoters (Trizzino et al., 2017).

Transcription of the *elovl5* genes are suggested to be regulated by sterol regulatory element-binding proteins (Srebps) and a liver X receptor (Lxr) (Carmona-Antoñanzas et al., 2016; Leaver et al., 2014). Srebps are transcription factors that regulate genes involved with lipid metabolism. These genes are regulated by Srebps through SRE binding sites, likely in cooperation with NF-Y (Carmona-Antoñanzas et al., 2016). Lxrs are ligand-activated transcription factors belonging to the nuclear receptor superfamily. They form a heterodimer with retinoid X receptor and together they bind to the liver X receptor response element (LXRE). These response elements are situated in the promoter sequence of the target gene. The Lxr/Rxr heterodimer has also been shown to activate transcription of Srebp-1c in mice (Yoshikawa et al., 2001) and *elovl5* has been identified as a Srebp-1c target gene (Qin et al., 2009). One hypothesis is that the TE insertion event in the *elovl5* promoters have caused differential Lxr/Rxr and Srebp binding affinities (Carmona-Antoñanzas et al., 2016).

Carmona-Antoñanzas et al. (2016) found that the *elovl5a* duplicate exhibits responsiveness to Lxr/Rxr transcription factors while *elovl5b* does not, and that this responsiveness might be due to an insertion that could also have be responsible for the greater size of the *elovl5a* promoter. The differences in the magnitude of Srebp response between promoters, however, could be attributed to a tandem duplication of SRE and NF-Y cofactor binding sites in the *elovl5b* promoter that the *elovl5a* promoter lack (Carmona-Antoñanzas et al., 2016). The *elovl5b* expression was shown to respond stronger to Srebp activation when both these sites were intact. The NF-Y/SRE topology observed in *elovl5a* is likely to represent the ancestral state of the *elovl5* gene (Carmona-Antoñanzas et al.,

2016). The hypothesis is that while one of the duplicates has retained the ancestral function, the other duplicate has been free to evolve and functionally diverge. The ancestral-like duplicate could be subject to stronger purifying pressure to maintain the ancestral regulation than its duplicate. The novel regulation however, could be a result of either adaptation and subsequent neofunctionalization or possibly by loss of purifying selection (neutral evolution) (Sandve et al., 2018). Although the diverging *elovl5* expression pattern between the ancestor, pike, and Atlantic salmon, is presumed to be a result of adaptation to new habitats, a previous study found indications that negative (purifying) selection pressure was the major evolutionary force acting on the salmon Elovl5 genes after their divergence from the Northern pike (Carmona-Antoñanzas et al., 2013). This negative selection pressure could explain the highly conserved enzymatic activity of the Elovl5 genes across salmonid species (Carmona-Antoñanzas et al., 2013).

1.7 Aims and objectives

The main aim of this thesis was to optimize a transfection protocol for transient transfection of primary hepatic cell culture of Atlantic Salmon. A secondary aim was to use this protocol to perform promoter reporter assays that could potentially give more insight into the mechanisms that drive the different expression patterns of two duplicates of Elovl5 in the liver.

2 Methods and materials

2.1 Transfection of primary hepatic cells – Workflow overview

The transfection optimization workflow included three main parts; (i) preparation of the primary hepatic culture, (ii) transfection, and (iii) analysis of reporter gene expression in transfected cells (**Figure 6**). Preparation of the primary hepatic cell cultures was done by liver perfusion with buffer containing collagenase. Collagenase enzymatically digests the extra cellular matrix (ECM) which leads to dissociation into single cells. The next step in the workflow varied dependent on the transfection technique being used, either chemical transfection by cationic lipid mediated transfection or electroporation (**Figure 6**). For chemical transfection, cells were seeded and left in 15°C or 20°C under ambient atmosphere for 24 hours to allow cells to recover and adhere to the culture well surface before transfection reagents were applied. When the cells were transfected by electroporation the cells were electroporated immediately after harvesting. Testing of transfection conditions was performed during step (ii). However, we found that optimization of step (i) also had a large impact on transfection efficiency. Transfection conditions were evaluated 24-120 hours post transfection either by comparing transfection efficiencies, which were determined by microscopy, flow cytometry, or luminescent signals using a plate reader (**Figure 6**).

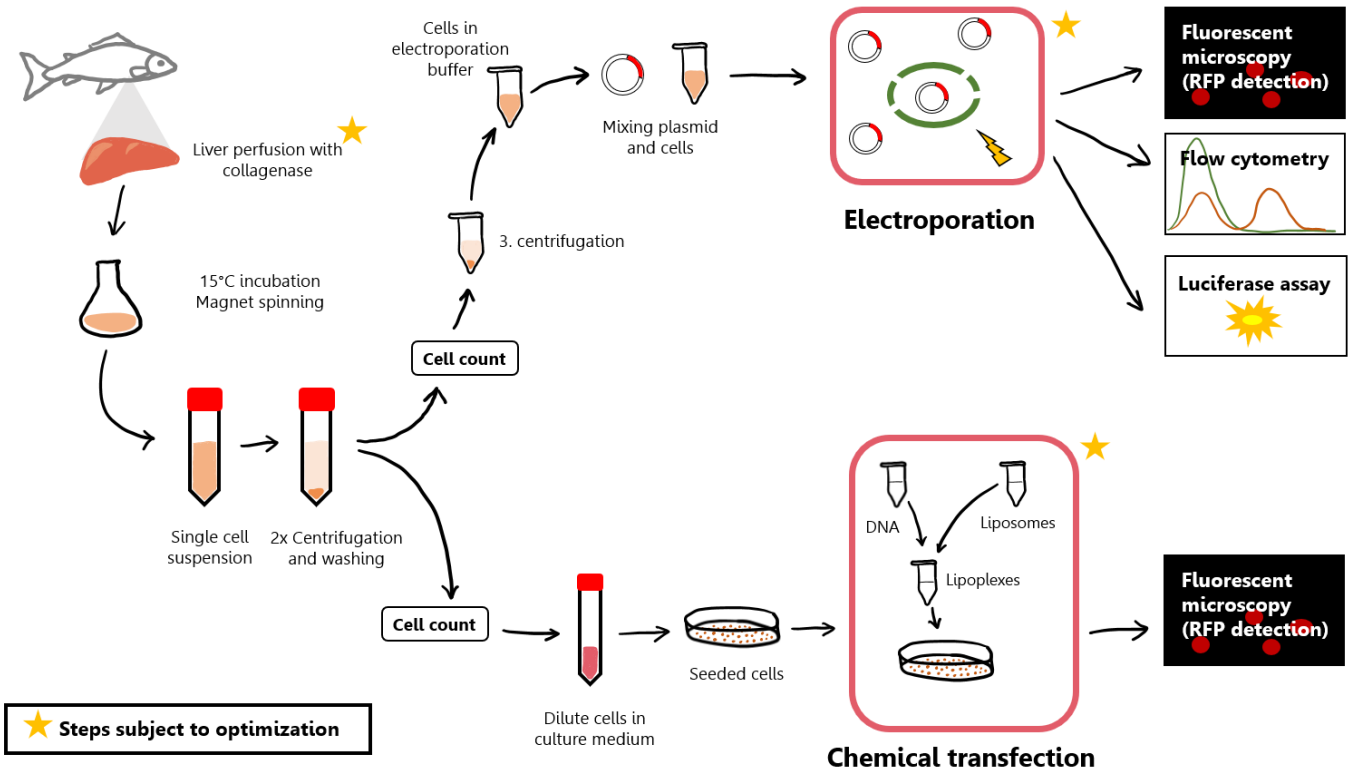


Figure 6. Schematic visualization of the workflow for transfection of primary liver cells. After isolation the cells were subject to either chemical transfection or electroporation. Transfection efficiency was evaluated by either fluorescent microscopy, flow cytometry or by luciferase assay. Both cell isolation and transfection were optimized.

2.2 Plasmids for transfection optimization

To determine transfection efficiency the liver cells were transfected with plasmid vectors containing two different reporter genes, expressing either red fluorescent protein (RFP) or Renilla luciferase (pGL4.75[hRluc/CMV]), respectively. Plasmid amplification was done by transforming chemically competent OneShot TOP10 *E. coli* cells using the Zero Blunt TOPO PCR Cloning kit (ThermoFisher, 2014) and growing transformants on LB agar plates containing either kanamycin or ampicillin for selective growth. Resulting colonies were used for inoculation of 10 mL liquid LB medium (Invitrogen, 12780052) in 50 mL falcon tubes added either 10 µl 50 mg/mL kanamycin (RFP plasmid resistance) or 10 µl 100 mg/mL ampicillin (*rluc* plasmid resistance). The tubes were kept at 225 rpm at 37°C overnight. Plasmid isolation was then performed using the QIAprep Spin Miniprep Kit from QIAGEN according to the manufacturers protocol except for a few changes to the elution step: The elution was performed using 30 ul Axiom water. Then the tube was placed in a 60°C waterbath for 1 minute before the sample was centrifuged for 1 minute. The sample was then applied anew to the column before it was heated and centrifuged again like described. Only plasmids with a purity (260/280 ratio) between 1.8 and 2.0 was used in these experiments. Their purity was determined with a NanoDrop 8000 spectrophotometer.

2.3 Fish

Atlantic Salmon used for isolation of the primary hepatocytes was from the Aquagen breeding stock. The size of the fish ranged from 22-33 cm in length and weight ranged from 120-493 grams. All fish used in the experiments were male.

2.4 Transfection optimization experiments

2.4.1 Chemical transfeciton – Transfection optimization I

2.4.1.1 Isolation of primary hepatic cells

Atlantic salmon was euthanized and sacrificed with a sharp blow to the head. The liver was then separated from the gut and placed in a cold sterile petridish on ice. Perfusion was performed by inserting a pipet tip without filter into the portal vein of the liver and flushing with ice cold wash buffer (50 mL 10X HBSS without Ca^{2+}/Mg^{2+} (Hanks Balanced Salt Solution, Gibco, 14185052), 1 mL 0.5 M ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, E7889), 5 mL 1 M HEPES (Gibco, 15630056), dH2O to a total of 500 mL, pH 7,4, stored at 4°C) using a peristaltic pump at a low rate for 20 minutes. Wash buffer was then replaced with collagenase buffer (20 mL 10X HBSS with Ca^{2+}/Mg^{2+} (Gibco, 14065049), 2 mL 1M HEPES (Gibco, 15630056), dH2O to a total of 200 mL, pH 7,5, stored at 4°C) with added type I collagenase type (Gibo, 17018029) to a final concentration of 150U/mL, and perfusion was continued for an additional 20 min. The liver was pulled into small pieces carefully shaken in the buffer to release single cells and then poured into an Erlenmeyer flask. The cells were then incubated for 1 hour at 15°C under ambient atmosphere on a magnet stirrer at low speed. Next, the cell suspension was poured through a 70 µm cell strainer into a 50 mL falcon tube and the strainer was carefully rinsed with Leibovitz's L-15 Medium (Gibco, 31415029) to give a uniform single cell suspension. When the cell suspension is made the cells are washed once, by centrifugation and resuspension in L-15. To produce a cell pellet, the cells were centrifuged at 50 g for 2 minutes at 4°C. The resulting cell pellet is very fragile; therefore, the supernatant was carefully removed before the pellet was dissolved in 5 mL pure cold L-15 to wash the cells, removing cell debris and other cells types native to the liver. The pellet proved difficult to dissolve, so the tube was tilted, swirled and very gently pipetted up and down to remove the clumps. The cells were then pelleted again at 50 g for 2 minutes at 4°C and the pellet was carefully dissolved in 1 mL cold culture medium with antibiotics (L-15 (Gibco, 31415029), 1% Pen-Strep (Gibco, 15140122), 1 % Amphotericin B (Gibco, 15290018) and 5% fetal bovine serum (Sigma-Aldrich, F7524). Cell count and cell viability were evaluated using trypan blue staining and Bio-Rad TC20 automated cell counter. The cells were diluted in culture medium with antibiotics on a sterile bench to give a concentration of $2 \cdot 10^5$ cells/cm^2, and then seeded in 24-well culture plates ($3.8 \cdot 10^5$ cells per well). The wells were coated 24 hours priot to cell seeding with 1x PEI polyethyleneimine (PEI, branched, Sigma, 408719) from a prepared 100x PEI stock (20 mg PEI in 20 mL borate buffer (1mg/mL), heated to 37°C for 10 min, stored at 4°C). Coating was done by aliquoting 1x PEI (20 mL of 10 ug/mL PEI by dissolving 200 µl 100x PEI stock in 19.8 mL

borate buffer in 0.1 M borate buffer (pH 8.4)) to each well and incubated for 1 hour, before the wells were washed twice with 1x HBSS with Ca^{2+}/Mg^{2+} (Gibco, 14065049). The plate was then left in room temperature for 24 hours. A volume of 0.5 mL of culture medium was used in each well. Cells were then incubated at both 15°C and 20°C under ambient atmosphere.

2.4.1.2 Cationic lipid mediated transfection using lipofectamine

In this initial chemical transfection experiment two chemical transfection reagents were used; Lipofectamine 3000 (Invitrogen) and Lipofectamine LTX (Invitrogen). These were tested using two different temperatures and three different transfection time points (**Figure 7**). To evaluate the transfection efficiencies, RFP was used as a reporter gene. For each lipofectamine reagent, two concentrations were tested, hereafter termed *high* and *low* (Lipofectamine 3000: 1.5 µl/1 µg DNA for high, 0.75 µl/1 µg DNA for low; Lipofectamine LTX: 5 µl/5 µg DNA for high, 2 µl/5 µg DNA for low). The cells were incubated at 15°C or 20°C. Cells incubated at 20°C were only tranfected using "Lipofectamine 3000 at *high* concentration" while cells at 15°C were using all conditions (**Figure 7**). Cells were transfected at three different times; As they were seeded (reverse transfection), after 24 hours and after 48 hours.

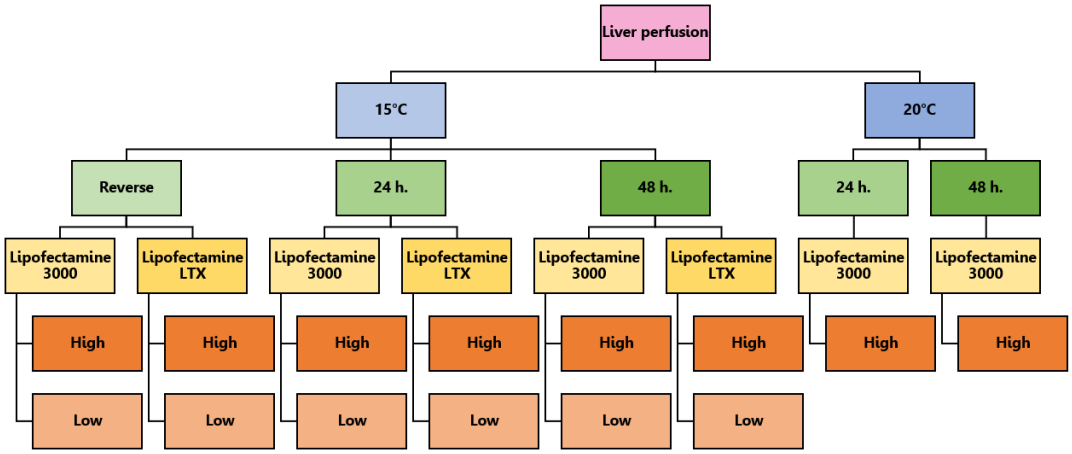


Figure 7. Experimental design for cationic lipid mediated transfection.

The transfections were performed according to the manufacturer's protocols (Lipofectamine 3000: ThermoFisher, 2016; Lipofectamine LTX: ThermoFisher, 2013) in duplicate. Culture media was changed before all transfections performed 24 hours and 48 hours post-seeding.

Transfection efficiency was evaluated by estimating fraction of RFP expressing cells using a fluorescent microscope. This was done 96 hours post-transfection for the cells transfected both 24 hours and 48 hours post-seeding, but 120 hours post-transfection for the cells that were reverse transfected. Fraction of RFP-expressing cells was calculated

based on number of live seeded cells (300 200 cells/well), except for two samples. For these samples the fraction of RFP-expressing cells was calculated based on live cells, which were counted by hemocytometer (see supplementary table 8).

2.4.2 Electroporation transfection – Transfection optimization II

2.4.2.1 Isolation of primary hepatic cells

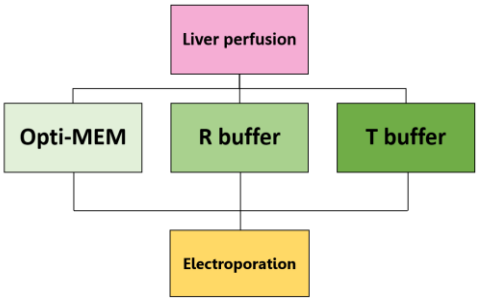
The isolation of hepatocytes was mostly performed the same way as in transfection optimization I, but with some minor changes to the protocol related to cell culture preparation and to accommodate electroporation. This time the liver was not separated from the gut before it was placed in a cold petridish on ice. The pipet tip used in the first optimization was substituted with a butterfly perfusion needle (Vacutest Kima s.r.l.). When the collagenase perfusion was done, the liver was detached from the gut. Cells in the strainer was rinsed using cool 1xHBSS without Ca^{2+}/Mg^{2+} (Gibco, 14185052), not L-15, and cells were pelleted by centrifugation at 100g for 5 minutes at 4°C. The supernatant was carefully removed and the cells were resuspended in 5 mL cold 1x HBSS without Ca^{2+}/Mg^{2+} (Gibco, 14185052). Small cell agglomerates that were difficult to dissolve were left in the solution. A second centrifugation was done at 100g for 5 minutes at 4°C, supernatant was removed, and the cells resuspend in 5 mL cold 1x HBSS without Ca^{2+}/Mg^{2+}. Small remaining cell clumps were again left in the solution. Hemocytometer and Trypan blue staining was used to determine viability and the cell count per mL. The cell suspension volume was split into three equal volumes in three microcentrifuge tubes. The three tubes were centrifuged at 100g for 5 minutes 4°C. The supernatant was carefully removed and the cells were resuspended in three different pre-cooled electroporation buffers; Opti-MEM (Opti-MEM I Reduced Serum Media, gibco)/Resuspension Buffer R (part of the Neon Transfection System Kit (ThermoFisher, 2014))/Resuspension Buffer T (also part of the Neon Transfection System Kit) at volume to give ~ $1 \cdot 10^7$ cells/mL.

2.4.2.2 Transfection by electroporation using the Neon transfection System

Optimization of electroporation transfection protocols were performed using the Neon Transfection System (ThermoFisher, 2014) and the Neon Transfection System Kit (**Figure 8**). Initially I tested three different electroporation buffers using the same electroporation program on all samples; 1600V, 10ms and 3 pulses; A new Neon protocol developed by the Roslin Institute (Gratacap et al., 2020). The RFP plasmid was used as reporter gene in all samples. Two negative controls were also included for each of the three conditions, one negative control where the plasmid was omitted and one control with plasmid but omitting the electroporation step. All three buffer conditions were done in duplicate.

Electroporation was performed according to the manufacturer's protocol (Invitrogen, 2014) for the 24-well culture plate format, designed for adherent cells using the 10 µl

Neon tips. Cell were suspended in electroporation buffer to give 1 x 10^5 cells per 10 µl Neon tip. The amount of plasmid DNA for each 10 µl Neon tip was set to 1 µg. Immediately after electroporation the cells were carefully distributed in wells with 0.5 mL culture medium without antibiotics (L-15 (Gibco, 31415029) + 5% fetal bovine serum (Sigma, F7524)) that were pre-cooled in 15 °C under ambient atmosphere. The pipet tips were used twice (for the same condition), washed by pipetting dH_2O a couple 2-4 times and saved to be reused. Fraction of RFP expressing cells (transfection efficiency) was to be evaluated manually 96 hours post transfection, using fluorescent microscopy, Trypan staining and hemocytometer, but due to reccuring technical issues with hemocytometer it was not possible to obtain reliable data. Fluorescent micsoscopy imaging was however done 24 hours post transfection and used to determine what electroporation buffer seemed to give the higher transfection efficiency.



Figur 8. Experimental design for first electroporation optimization.

2.4.3 Electroporation transfection – Transfection optimization III

2.4.3.1 Isolation of primary hepatic cells

The isolation of hepatocytes was performed as described, with some minor changes from section 2.4.2.1. When the wash buffer perfusion was done, the liver, still attached to the gut, was moved to a cold sterile petridish. This was done in a way so that the chelating agent EDTA in the wash buffer would not come in contact with the collagenase buffer which can inhibit the collagenase activity. This time fresh collagenase was added to the collagenase buffer, which had a lighter brown color, compared to the one used for the previous transfections, which was dark brown in color. When the collagenase perfusion was done, the liver was detached from the rest of the gut and moved to another petridish before dissolving the tissue, resulting in a single cell solution. Cell count and viability were determined manually using a hemocytometer and Trypan blue staining. Like the previous transfection (electroporation optimization I), the cells were centrifuged a third time before resuspension in electroporation buffer to give ~$1 \cdot 10^7$ cells/mL. Resuspension Buffer R (ThermoFisher) was used for all transfections.

2.4.3.2 Electroporation using a 24-well optimization protocol

Transfection optimization experiment III focused on testing different_electroporation programs built into the Neon Transfection System (see supplementary table 2 and **Figure 9**), in effect testing the combinatorial effect of voltage, pulse width and number of pulses. In this experiment transfection efficiencies were quantified transfection using renilla luciferase as a reporter gene (pGL4.75[hRluc/CMV]). The same plate format, culture medium, Neon tips, plasmid DNA amount (µg) and cell number/well as in the first electroporation optimization was used (2.4.2.2). The experiment was performed according to the manufacturer's protocol (Invitrogen, 2014) without replicates. After 24 hours the electroporation programs were compared by performing a luciferase assay using the Dual-Luciferase Reporter Assay System kit from Promega with the 96-well opaque plate format and a SpectraMax M2^e plate reader. The results were evaluated based on renilla luciferase signal. The luciferase assay was performed according to the manufacturer's protocol (Promega, 2015).

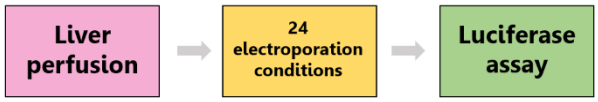


Figure 9. Experimental design for second electroporation optimization.

2.4.4Electroporation transfection – Transfection optimization IV

2.4.4.1 Isolation of primary hepatic cells

The hepatocyte isolation was performed the same way as in transfection optimization III (section 2.4.3.1). Cell concentration and viability were evaluated using Trypan blue staining and a hemocytometer, like with optimization III and II, before the number of cells needed for the experiment was centrifuged one last time (this time the last centrifugation was shortened to 1 minute) and resuspended at volume to give ~1x10^7 cells/mL).

2.4.4.2 Further comparisons of electroporation programs

In this round of optimization we replicated the best performing electroporation experiments from 2.4.3, and compared these to the Neon protocol developed by the Roslin Institute (Gratacap et al., 2020) (**Figure 10**). Two negative controls conditions were also included, *no plasmid* and *no electroporation*. All protocols were tested in triplicate and the RFP reporter plasmid was used for all transfections. The plasmid DNA volume was 1 µg per 10 ul Neon tip. This time flow cytometry was used to evaluate the transfection efficiency of the different electroporation programs (see section 2.4.4.3 for details) 48 hours post transfection.

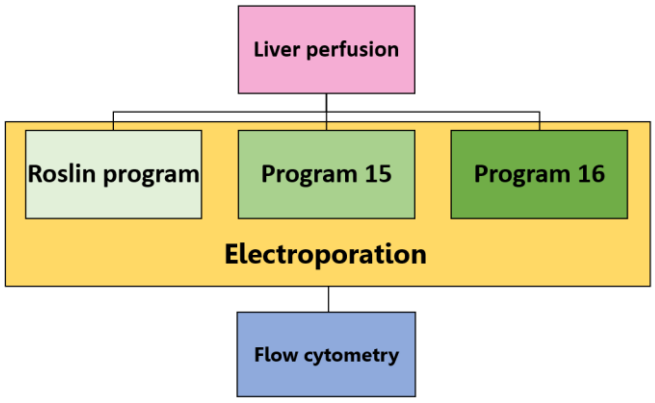


Figure 10. Experimental design for final electroporation optimization.

2.4.4.3 Transfection efficiency estimation by flow cytometry

Transfections done in the transfection optimization IV experiment were used for transfection efficiency estimation using flow cytometry. Forty-eight hours post transfection the culture medium was discarded, and the monolayer of cells was washed twice with 0.5 mL 1x HBSS without Ca^{2+}/Mg^{2+} to remove all traces of fetal bovine serum (F7524, Sigma) used in the culture medium. Then 300 µl Trypsin was added to each well and the coated cells were allowed to incubate until the cells detached from the well surface, about 7 min. 20 ul FBS (F7524, Sigma) was added to each well to inactivate the trypsin. The cell samples were then pelleted at 100g for 10 min. at 15°C and subsequently washed in 1 mL Hanks. The cells were pelleted again at 100 g for 10 min. at 15°C and resuspended in 50 ul already filtered PBS (filtered through 0.2 µm strainer) without Ca^{2+}/Mg^{2+}. The resuspended cells were then filtered with a 70 µm cell strainer. The cell suspension samples were checked for clumps under microscope to avoid clogging of the flow cytometer instrument.

The samples were run using a CellStream Flow Cytometer and PBS (without Ca^{2+}/Mg^{2+}) as sheath fluid. A density plot of the detected side scatter vs. the detected forward scatter was made to gate the target live hepatocyte cell population using a negative control sample of un-transfected cells before running electroporated samples. The red fluorescent protein in electroporated samples was excited by a 561 nm laser, and 611 nm emission was detected using the 611/35 channel of the instrument. The CellStream Analysis 1.2.1. program was used to process the flow cytometry data and determine the transfection efficiency, which in this case is given as the percentage of red fluorescent cells of the gated cells (viable hepatocytes). The CellStream Analysis 1.2.1. program was used to analyse the flow cytometry data.

2.5 Elovl5 promoter reporter assay experiments

2.5.1 Constructing plasmids for Elovl5 promoter reporter assay

A secondary aim of this master project was to use the new hepatocyte primary cell system to perform a PRA comparing the *elovl5* gene duplicates in salmon. We wanted to test four different PRA-constructs: The two WT promoters from *elovl5a* and *elovl5b* (**Figure 11**) and two synthesized synthetic promoters from the same genes based on the open chromatin profiles from ATAC-seq data (**Figure 12**). These synthetic promoters contain the most likely functional CRE's that regulate the *elovl5* gene duplicates.

2.5.1.1 Genomic DNA isolation

To generate the Atlantic Salmon Elovl5a WT promoter construct genomic DNA was isolated by organic extraction. Liver tissue was homogenized using a glass douncer and the hepatocytes were then lysed by proteinase K which, enzymatically digests protein. To remove cell membrane lipids 1% SDS (detergent), was added, together with EDTA and 10 mM Tris-HCl and left for 4 hours at 50°C. Next, 1 mL 1x phenol:cloroform was added at the volume of the lysate in phase-lock tubes in a fume hood before the tubes were inverted for 10 minutes to form a homogenous solution. The samples were then centrifuged for 10 minutes at **175543g**. The upper phase containing the DNA was pipetted into a clean Eppendorf tube. The DNA was precipitated with 0.24M NaCl and ice cold 99% ethanol. The DNA became visible at the interphase and was transferred into a new Eppendorf tube before it was then pelleted by centrifugation for 10 min. at 10 000 rpm and subsequently washed with 500 µl 70% EtOH. The pellet was dried by leaving the lids open for a few minutes and re-dissolved in 50 µl Axiom water 96 and the gDNA concentration was measured with a Qubit fluorometer before it was frozen at -20°C.

2.5.1.2 PCR amplification of WT promoters

The Elovl5 WT promoter region was amplified by PCR using primer sequences from Carmona-Antoñanzas et al., 2016 (see supplementary table 4 for PCR primer sequences). In this study the primers were designed so that the upstream boundary of the WT promoter region was determined by a conserved SacI site observed in both the Elovl5a and Elovl5b duplicates. As described by Carmona-Antoñanzas et al., 2016, the WT promoter constructs contains upstream untranscribed sequence, TSS, a 5′ UTR non-coding exon (forms part of the mRNA, but is not translated) and an ATG initiation codon in the boundary of the second exon (where the *elovl5* gene starts) (**Figure 11**). The elovl5a WT promoter construct should be 4913 bp, and the elovl5b WT promoter construct should be 3143 bp.

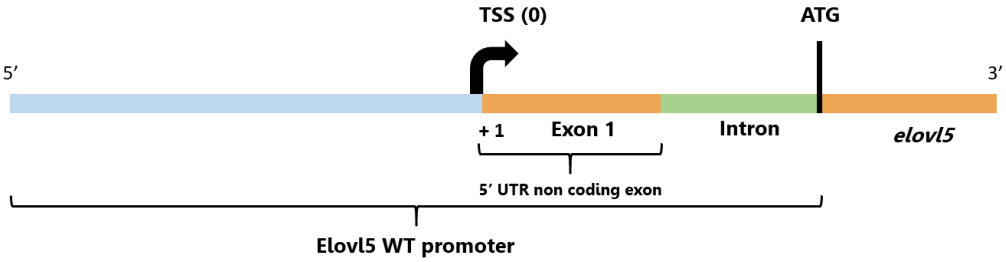


Figure 11. Schematic figure of the *elovl5* WT promoters. A schematic figure showing the WT promoter constructs defined by Carmona-Antoñanzas et al., 2016. The upstream untranscribed sequence in light blue, the TSS, the 5'UTR non-coding exon in orange and an ATG initiation codon at the start of the second exon marking the start of the Elovl5 coding sequence.

The PCR of the WT promoters was done using Invitrogen Platinum SuperFi PCR Master Mix (ThermoFisher, 2017) according to the manufacturer's protocol for 25 µl reactions, using 20 ng Atlantic Salmon template gDNA per reaction. Annealing temperature was set to 60°C and 30 cycles were run. The amplification of the elovl5b WT promoter failed, and additional PCRs were run with inclusion of 2% and 5% DMSO (D2438-5X10ML, Sigma) at 52°C and 55°C. The PCR product samples were run on a 1% agarose gel to verify PCR product size.

2.5.1.3 Cloning of WT promoter regions

The WT promoter amplicons were cloned into a pCR-BluntII-TOPO vector with compatible blunt ends, and then used for transformation of chemically competent OneShot TOP10 *E. coli* cells using the Zero Blunt TOPO PCR Cloning kit (ThermoFisher, 2014). The empty topo vector has topoisomerase I recognition sites between which the insert is ligated. The cells were then plated on LB agar plates with kanamycin to select for transformants.

Colony PCR was then used to determine the presence of transformed cells containing vectors with the correct insert DNA. The mastermix was made according to the AmpliTaq Gold DNA Polymerase Manual for 25 µl reactions. The pCR-Blunt II-TOPO vector containing the *elovl5* WT promoter construct also contains M13 forward and reverse primer sites that can be used for PCR screening (se supplementary table 5 for colony PCR primer sequences). The colony PCR was run at 55°C with 35 cycles.

When colonies with correct insert DNA were identified, 10 mL liquid LB medium (Invitrogen, 12780052) with 10 µl 50mg/mL kanamycin was inoculated and kept at 225 rpm at 37°C overnight for plasmid isolation. The topo-*elovl5*(wt) plasmid was purified using the QIAprep Spin Miniprep Kit from QIAGEN, 2019, according to the manufacturer's protocol, except for some changes made to the elution step as described above. Bacterial glycerol stocks (25% glycerol) were also made and stored at -80°C.

2.5.1.4 Design of synthetic elovl5 promoters

The two synthetic promoter constructs were designed based on results from an Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) experiment conducted in a different project (**Figure 12**) and not described here. In brief, this experiment provided information on which regions of the Atlantic Salmon genome that are open chromatin, and therefore assumed to be accessible for transcription factors to bind and regulate nearby genes. The *elovl5* promoters contained four and three ATAC-seq peaks, i.e. open chromatin regions in the promoter, in the *elovl5a* and *elovl5b* promoters, respectively. The open chromatin regions in the promoter regions were manually identified and extracted as fasta sequences. These fasta-sequences were concatenated to give the two elovl5 ATAC peak promoter constructs (1154 and 1083 bp respectively) and sent for synthesis at Genscript, Norway. Constructs were delivered in 2710 bp pUC57 vectors, from now on termed pUC57-peaks-*elovl5a* (3864 bp) and pUC57-peaks-*elovl5b* (3793 bp). The pUC57 contained HindIII and KpnI restriction sites later used to cut the promoter constructs from the plasmid.

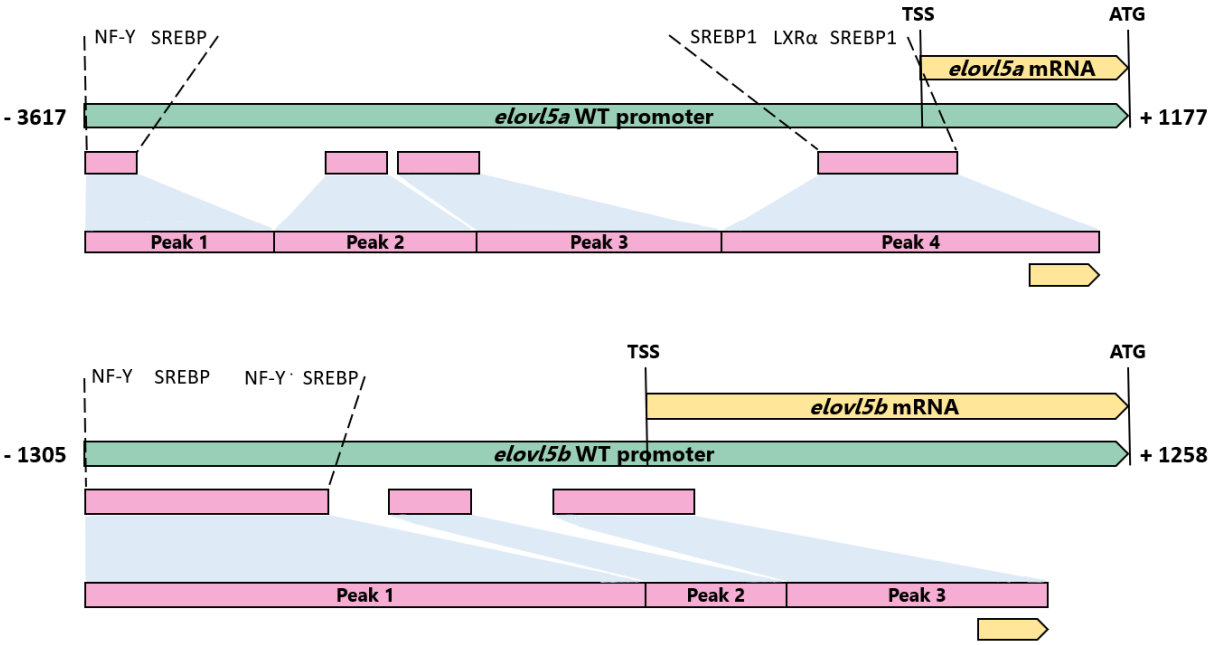


Figure 12. Schematic figure of the *elovl5* synthetic promoter constructs. ATAC-seq peaks indicating open chromatin are concatenated to constitute the two synthetic promoter constructs. The start of the elovl5 mRNA marks the position of the TSS. The ATG initiation codon marks the end of the WT constructs and the translation start point (the ATG is replaces by luciferase ATG initiation codon during cloning). CREs suspected to be causing differential expression of the *elovl5* duplicates in Atlantic salmon liver by Carmona-Antonanzas et al., 2016, (NF-Y, SREBP, SREBP1 and LXRα) are included in the peaks.

2.5.1.5 Construction of elovl5 promoter reporter assay vectors

The expression vector (pGL4.10[luc2]) was linearized by digestion using two pairs of restriction enzymes, HindIII-HF and KpnI-HF in addition to SacI-HF and XhoI (all from NEB). The resulting sticky ends were compatible with both the WT and synthetic promoter constructs. The pUC57-peaks-*elovl5a* and pUC57-peaks-*elovl5b* plasmids were then digested by HindIII-HF and KpnI-HF and the topo-*elovl5*(wt) plasmids were digested

by SacI-HF and XhoI. All digestion reactions were set up using: 2 µg plasmid DNA, 20 U of each restriction enzyme, 5 µl 10x CutSmart Buffer (NEB, B7204S), dH_2O to 50 µl. The reactions were incubated at 37°C overnight. Three controls were made per digestion reaction; Two controls with only one of the two restriction enzymes in addition to undigested control. After 24 hours the digestion reactions were run on a 1% agarose gel before the linearized expression vector (pGL4.10[luc2]) and *elovl5* constructs were extracted from the gel. The DNA from gel bands were purified using the QIAquick Gel Extraction kit (QIAGEN, 2018) according to the manufacturer's protocol.

The purified promoter constructs were cloned into the promoterless Firefly luciferase expression vector (pGL4.10[luc2]) (**Figure 13**). The ligation reactions were as follows: 100 ng linearized pGL4.10[luc2] vector, insert DNA (using a 3:1 insert to vector ratio), 2 µl 10x Ligase Buffer with 10 mM ATP, 1 µL T4 DNA Ligase and dH2O to 20 µl. The reactions were incubated at 4°C overnight. One negative control with no insert was made for each of the ligation reactions. Chemically competent OneShot *E. coli* cells were transformed with ligations reactions according to the Zero Blunt TOPO PCR Cloning kit protocol for OneShot Chemical Transformation (ThermoFisher, 2014) and grown on LB agar plates with ampicillin. 10 mL LB medium with added ampicillin to working concentration (100 µg/mL) was inoculated with transformant colonies, and plasmids were purified using the QIAprep Spin Miniprep Kit from QIAGEN.

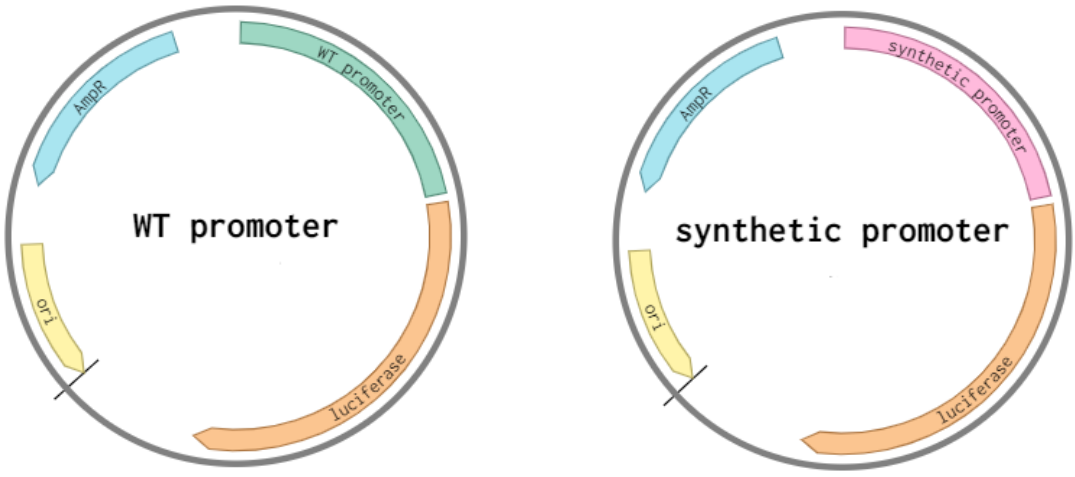


Figure 13. Elovl5 promoter constructs in luciferase based expression vector for promoter reporter assay. The Elovl5 WT (green) and synthetic (pink) promoter constructs in the pGL4.10[luc2] expression vector. The promoters are situated upstream of the luciferase gene (orange).

The ligation reactions were evaluated by control digestions of the plasmids to check for the presence of inserts of correct size. The ligation of the *elovl5a* ATAC synthetic promoter into pGL4.10[luc2] failed, and a new ligation was run for this reaction only. This time a dephosphorylation of the linearized expression vector was done before the ligation reaction, using FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher, EF0651). The reaction was set up as follows: 500 ng linearized expression vector, 0.5 U FastAP Thermosensitive Alkaline Phosphatase, 1 µl 10x FastAP reaction buffer and nuclease free water to 10 µl. The reaction was run for 10 min. at 37 °C and inactivated by 5 min.

incubation at 75°C. This ligation was otherwise performed as described above, including a new control digestion.

When ligation was done the vectors were sent off to Eurofins Genomics (Moss, Norway) for verification by Sanger sequencing. Sequencing primers were designed (see supplementary table 6 for sequencing primers) and samples prepared according to LightRun sample submission instructions (Eurofins Genomics, 2020).

2.5.2 Transfection with WT and synthetic *elovl5* promoter constructs

Primary hepatic cell culture was prepared the same way as in electroporation optimization III, except that the perfusion was performed faster, ~12 min. per perfusion buffer, due to the portal vein being located immediately. For the promoter reporter assay the cells were transfected with the WT promoter and the synthetic promoters in the pGL4.10[luc2] expression vector. The same cells were co-transfected with *renilla luciferase* plasmids (pGL4.75[hRluc/CMV]). The Renilla expression was used as a bioluminescence baseline to normalize for well-to-well variability that can occur during the luciferase assay. The *rluc* transcription is driven by a strong CMV promoter ensuring constitutive expression. The co-transfection was done at a 1:1 plasmid ratio (see table.. for details). RFP was used as a positive control for the transfection. Two types of negative controls were also included: One where both plasmid and electroporation were omitted, and one transfected with an empty pGL4.10[luc2] vector. All five conditions were done in triplicate in 24-well culture plates, using the same total DNA amount, cells/10 µl Neon tip and culture medium as in the previous electroporations. The best performing electroporation program from transfection optimization III was used for this electroporation (1400V, 20ms, 2 pulses).

Table 1. Plasmids per 10 µl Neon tip in reporter assay transfections. For the promoter reporter assay cells were transfected with the WT and synthetic (atac) promoter constructs. Six conditions were used in the experiment, and four of these were co-transfections (*) with both firefly and renilla luciferase plasmids at a 1:1 ratio. The pGL4.10[luc2] vector was used for the empty vector condition.

	Conditions					
	No electroporation/plasmid	Empty vector*	RFP	*elovl5a-atac**	*elovl5b-atac**	*elovl5a*-WT*
Empty pGL4.10[luc2]		0.5 µg				
RFP			1 µg			
***elovl5a* s.p.**				0.5 µg		
***elovl5b* s.p.**					0.5 µg	
***elovl5a* WT p.**						0.5 µg
rLuc		0.5 µg		0.5 µg	0.5 µg	0.5 µg

2.5.3 Dual-Glo Luciferase Assay

Forty-eight hours post transfection luminescence was measured by a BioTek Synergy H1M plate reader. The Dual-Glo Luciferase Assay System was used according to the manufacturer's protocol (Promega, 2015). The culture medium in the plates was changed to DMEM (Sigma, D6429) before starting the lysis with the addition of the of Dual-Glo Luciferase Reagent.

Background was subtracted from the luciferase read data by finding the average of the un-electroporated sample read data and subtracting that from the firefly and renilla readings separately. To account for well-to-well variability caused by factors such as differences in cell number, live cell number and transfection efficiency, the values were normalized by calculating the firefly:renilla ratio. One sample replicate reading was not included in the dataset due to a pipetting error (*elovl5a*-WT, replicate 3).

3 Results

3.1 Primary cell isolations

All wash buffer perfusions were carried out so that the liver turned a yellow-ish color (**Figure 14**). Perfusion times varied from 12-20 min. After the collagenase perfusions, single cell suspensions were prepared. To remove other cell types and cell derbis, the suspensions were centrifuged, and the cells washed. In chemical transfection these cells were seeded directly after this and transfected at a later point. For electroporation these cells were transfected right away. In transfection optimization I (2.4.1.1), a visible layer of blood cells appeared on top of the cell pellet after the centrifugation. The same thing was observed with all the subsequent perfusions. This layer was not, however, as pronounced in the pellet prepared for the final transfection for the promoter reporter assay (2.5.2, and **Figure 14**).

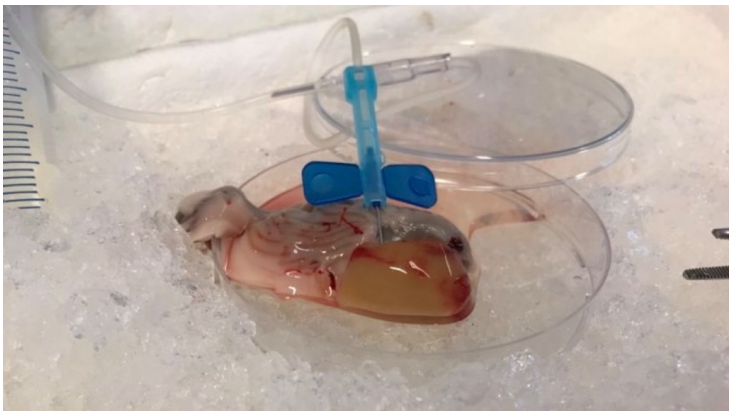


Figure 14. Perfusion of Atlantic Salmon liver for promoter reporter assay. A two-step collagenase perfusion with peristaltic pump and butterfly perfusion needle. Image from the transfection performed in connection with the promoter reporter assay.

During the two first cell culture preparations (section 2.4.1.1 and 2.4.2.1), some cell agglomeration was observed after centrifugation. These clumps were difficult to dissolve completely. Consequently, a few of these were left in what should have been single cell suspensions. After some changes were made to the cell culture preparation protocol during transfection optimization III (2.4.3.1, 2.4.4.1), cell agglomeration was almost diminished. Cell concentrations and viabilities of the single cell suspensions are shown in **Table 2**.

Table 2. Cell concentrations and viability measured manually by hemocytometer and microscopy. Note: Data from optimization I were collected with a Bio-Rad TC20 automatic cell counter.

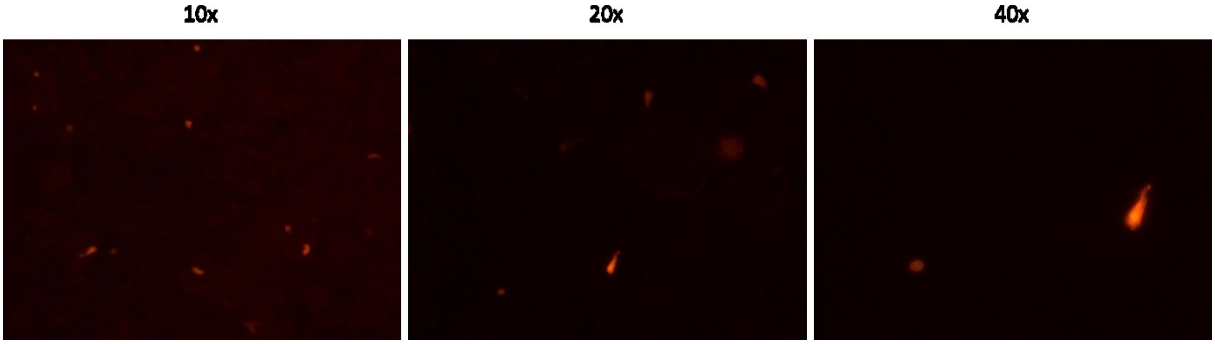
	Transfection experiment				
	I	II	III	IV	PRA
Total cells/mL	$12{,}09 \cdot 10^6$	$7{,}88 \cdot 10^5$	$29{,}2 \cdot 10^6$	$25{,}8 \cdot 10^6$	$24{,}2 \cdot 10^6$
Live cells/mL	$15{,}3 \cdot 10^6$	$6{,}3 \cdot 10^5$	$19{,}86 \cdot 10^6$	$21{,}9 \cdot 10^6$	$21{,}8 \cdot 10^6$
Viability (%)	79	80	68	85	90

In the first transfection optimization (section 2.4.1) culture well adherence was checked by microscopy for cells that were transfected after seeding (after 24 and 48 hours). For all subsequent electroporations, well surface adherence was checked 24 hours after electroporation. In all experiments most cells seemed to have adhered.

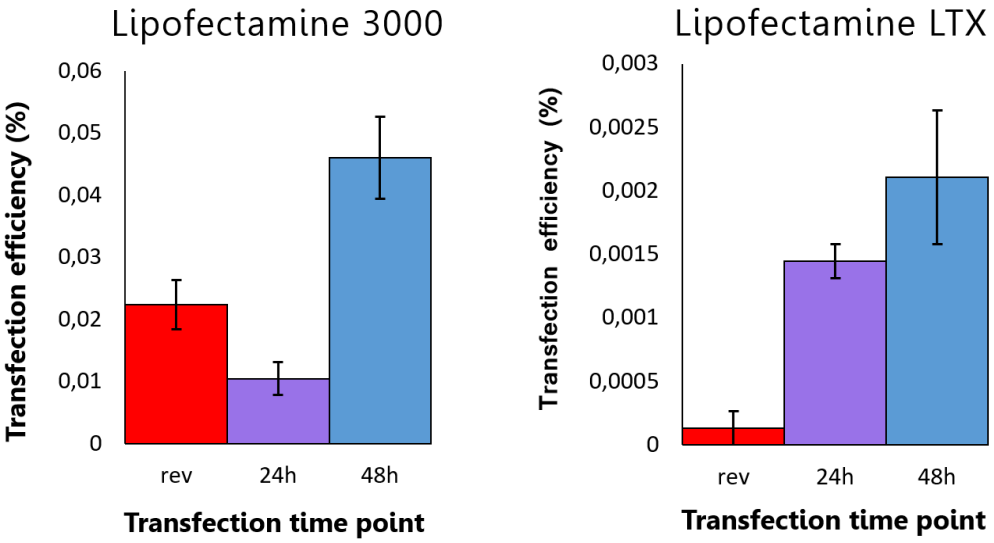
3.2 Low transfection efficiencies with chemical transfection

For the chemical transfection (section 2.4.1.2) several variables were tested; Two lipofectamine reagents, lipofectamine reagent concentrations (*low*/*high*), transfection time after seeding (reverse transfection, 24 and 48 hours) and incubation temperature (15°C and 20°C). To quantify transfection efficiency for chemical transfection the fraction of RFP expressing cells of the total live seeded cells was estimated. None of the chemical transfection protocols we tested produced estimated transfection efficiencies above 1% (see supplementary table 8 and example in **Figure 15 A**). Yet, we did observe differences in transfection efficiencies between the conditions (Figure 15 B-C).

A



B



C

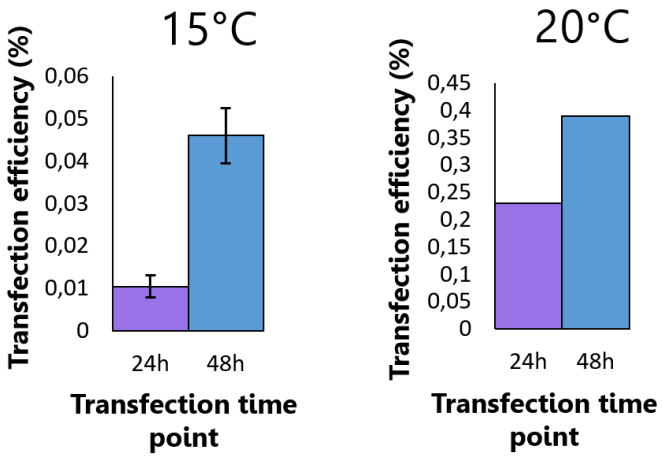


Figure 15. Results from chemical transfection using lipofectamine. **A:** RFP expressing cells after transfection using Lipofectamine 3000 at high concentration at the time of seeding, incubated at 15°C. **B:** Transfection efficiencies from using Lipofectamine 3000 compared to Lipofectamine LTX (at incubation temperature 15°C only). Transfection efficiencies from all transfection time points (rev., 24h and 48h) are shown. Only high concentration data are included due to missing data from low concentration samples. Bars show standard deviation. **C:** Differences in transfection efficiencies between incubation temperature 15°C and 20°C for cells transfected with Lipofectamine 3000 at high concentration 24 and 48 hours post transfection. Bars show standard deviation. Note that two samples were lost from the 20°C condition, consequently there are no replicates from this condition and no standard deviation is shown for this condition.

Firstly, the number of cells expressing RFP was higher at all transfection times using Lipofectamine 3000 compared to using Lipofectamine LTX (**Figure 15 B-C**). Of the three transfection time points, 48 hours post transfection gave higher transfection efficiencies (**Figure 15 B-C**). The results also suggest that the 5°C incubation temperature difference (15°C vs. 20°C) increases transfection efficiency (**Figure 15 C**) by 2-5-fold.

Unfortunately, the low concentration transfections were not included in the 24-hour samples and this not included in **Figure 15**. Nevertheless, the low concentration transfection efficiencies from the other two transfection times (rev. and 48 h) were very low, indicating that low concentration chemical transfection protocols performed worse than high concentration protocols.

3.3 Improved transfection with electroporation

In the first electroporation optimization (section 2.4.2.2) three electroporation buffers were compared and RFP was used as reporter gene. Cells transfected with the three buffers differed pronouncedly in RFP-cell expression, with the buffer R showing higher numbers of RFP-expressing cells (**Figure 16**). Due to a technical issue with the hemocytometer we were not able to count cells and thus failed to get good estimates of transfection efficiencies. Nevertheless, qualitative assessments by visual inspection of the samples 24 hours post transfection supported best transfection efficiency using buffer R, and a radically improved transfection efficiency compared to chemical transfection (see **Figure 16**, **Figure 15** and supplementary table 8).

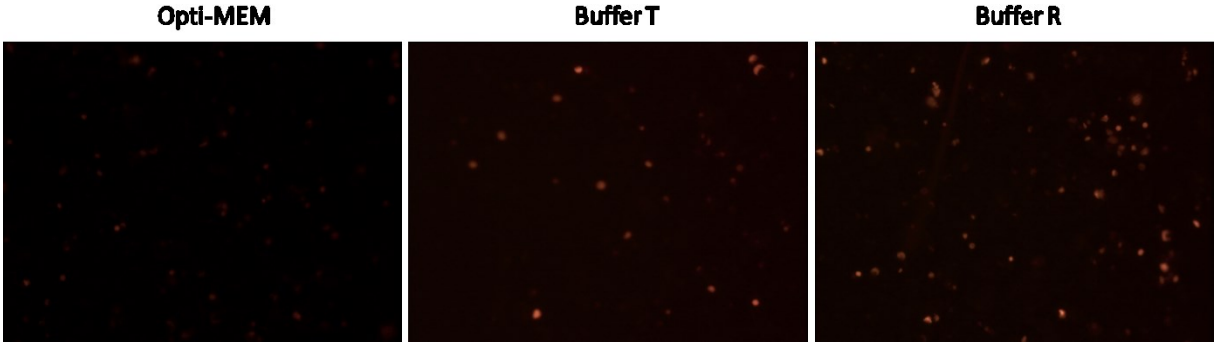


Figure 16. RFP expression after the first electroporation optimization. RFP expressing hepatocytes 24 hours post transfection at 10x. Cells were electroporated in three different electroporation bufferts (Opti-MEM, T-buffer and R-buffer). Negative controls are omitted from the figure but contained no fluorescent cells.

3.4 Different electroporation programs results in highly variable transfection efficiencies

In transfection optimization III (section 2.4.3.2) we continued using the resupension buffer R as electroporation buffer and tested 24 different electroporation programs, using renilla luciferase as reporter gene to compare transfection efficiencies. The RLU signal varied up to 2000-fold between the electroporation programs (**Figure 17**), with four programs giving RLU signal of >500. It should be noted that this experiment was performed without replicates, and that independent assessment of two promising programs (5 and 16) is described in the section below.

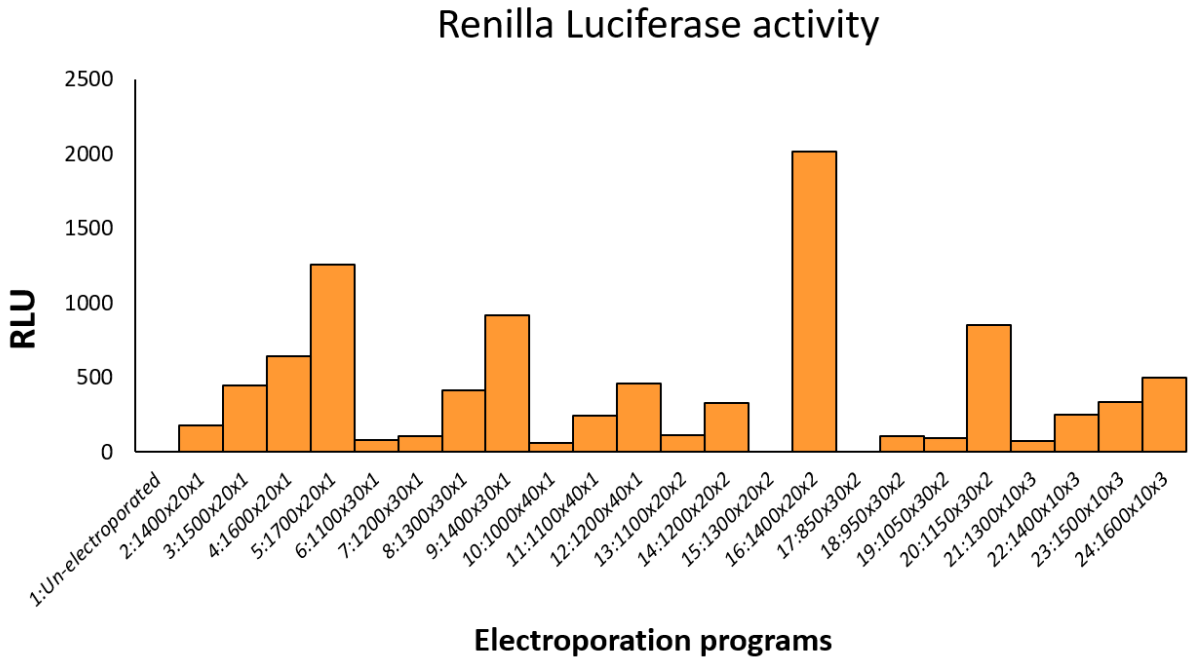


Figure 17. Renilla Luciferase activity in cells electroporated with 24 different electroporation programs. The Renilla signal is measured in relative light units (RLU). Background signal from un-transfected sample (program 1) is subtracted.

3.5 Comparing high performing protocols with a new protocol from The Roslin Institute

In this experiment the aim was to compare two of the best performing electroporation programs (section 2.4.4.2; programs 5 and 16) with a transfection protocol developed by the Roslin Institute. Visual inspection of fluorescent images taken 48 hours after transfection indicated that program 16 produced the-highest transfection efficiency (**Figure 18**), supporting our previous results (**Figure 17**).

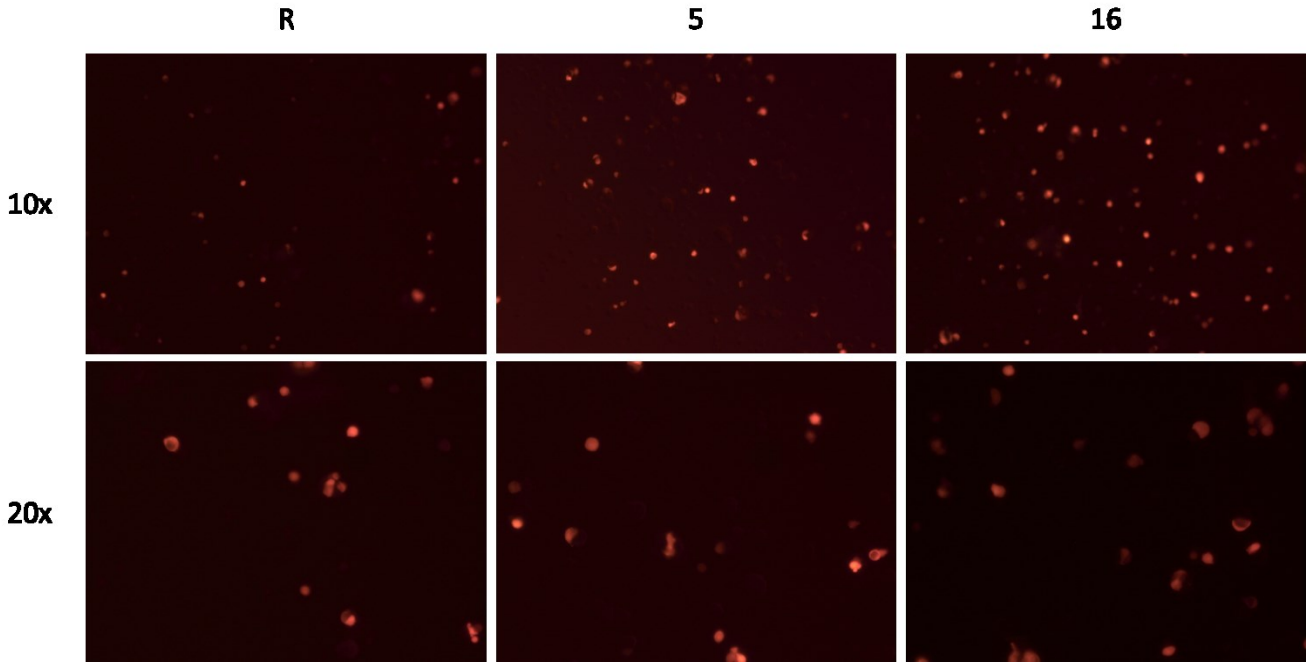


Figure 18. RFP expressing cells after electroporation. Images of RFP expressing cells 48 hours after transfection at 10x and 20x. Cells electroporated with three different electroporation programs: Program R (1600V, 10ms, 3 pulses), program 5 (1700V, 20ms, 1 pulse) and program 16 (1400V, 20ms, 2 pulses). Negative controls are omitted from the figure but contained no fluorescent cells.

Transfection efficiency measured by flow cytometry

Transfection efficiencies were also determined using a CellStream Flow Cytometer by estimating the proportion of the hepatocyte cell population emitting fluorescence at a wavelength corresponding to red fluorescent protein (transfection efficiency). The estimated transfection efficiency for the three protocols are shown in **Figure 19**. Program 16 displayed the highest transfection efficiency (30%), with the Roslin protocol and program 5 showing only slightly lower efficiencies. These results are consistent with more qualitative transfection efficiency estimates from fluorescent microscopy (**Figure 18**) as well as the renilla luciferase assay experiment (**Figure 17**).

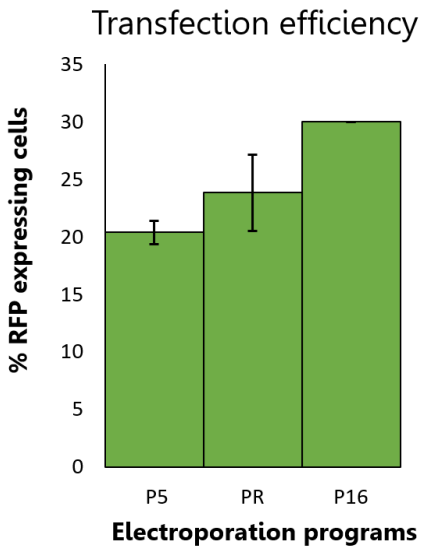


Figure 19. Transfection efficiency from flow cytometry analysis. Bars show standard deviations. Data from two samples from electroporation program 16 were lost during the calibration of the flow cytometer instrument (replicate 2 and 3), thus no standard deviation is shown for this condition.

3.6 *elovl5* promoter reporter assay

As a proof of principle, we aimed to use the optimized transfection protocol to conduct a promoter reporter assay. Our initial aims of this experiment were first to reproduce results from Carmona-Antoñanzas et al. (2016) as a proof of principle, and second to improve our understanding of cis-regulatory control of the two *elovl5* gene duplicates by also conducting reporter assays using synthetic promoters containing only CRE's predicted to be bound by transcription factors (Methods and materials 2.5.1.4).

3.6.1 Construction of plasmids for promoter reporter assay

The WT promoter constructs were amplified from genomic DNA with PCR, with predicted PCR product sizes of 4913 bp (*elovl5a*) and 3143 bp (*elovl5b)*. Running the PCR products on a 1% agarose gel showed that we recovered the *elovl5a*-WT promoter amplicon with expected size, however the *elovl5b* PCR reactions gave multiple bands.

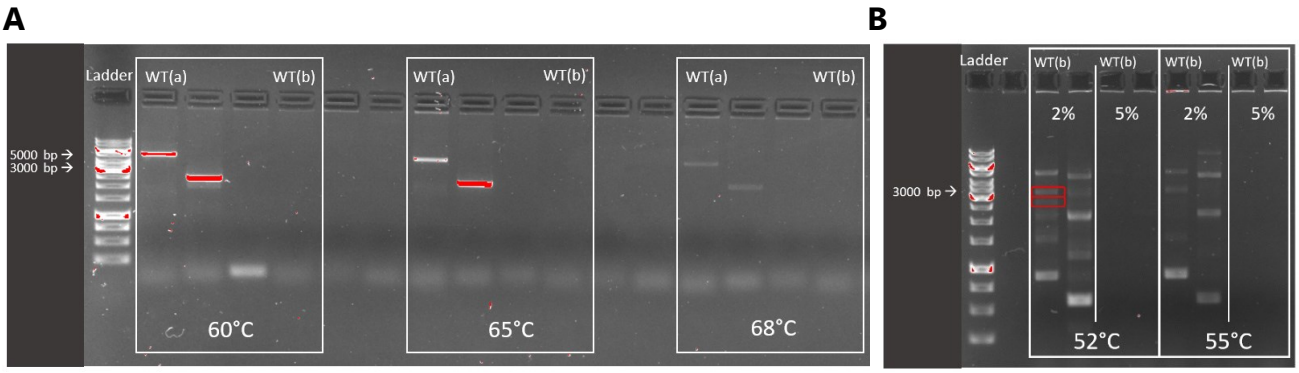


Figure 20. PCR reactions run on 1% agarose gel. **A:** Initial PCR products run at 60°C, 65°C and 68°C with 30 cycles. Gel was run at 70V for 45 minutes. *elovl5a*-WT construct was verified by a band of ~5000 bp. No bands are visible for the *elovl5b*-WT promoter construct at ~3000 bp. **B:** Later PCR products run at 52°C and 55°C, with addition of 2% and 5% DMSO. PCR produced several bands, of which two (marked red) were possible candidates to be the *elovl5b*-WT contructs based on their size (~3000 bp). Both gels were run at 70V for 45 minutes and bands were compared to the 1kb GeneRuler DNA ladder.

The two bands corresponding to the size of the *elovl5b-WT* construct had to be extracted from the gel before they could be used further. The shorter PCR product was termed *elovl5b*-WT-S and the longer was termed *elovl5b*-WT-L. Following the OneShot TOP10 cloning of the three amplicons into *E. coli* cells, a colony PCR was run to confirm transformants with correct insert. PCR products showed faint bands for colony 13 and 17 at expected size of the *elovl5a*-WT promoter construct. Neither of the *elovl5b*-WT colony PCRs produced any visible bands. Due to time limitations we unfortunately had to discard the *elovl5b*-WT construct from the following reporter assay experiments.

Figure 21. Colony PCR run on 1% agarose gel. **A:** *elovl5a*-WT colony PCR products from colonies 11-17 run on agarose gel to check for transformants with insert of correct length. Colony PCR was run at 55°C with 35 cycles. Faint bands at ~5000 bp corresponding to colony 13 and 17 verified that these colonies are transformed with vectors containing the *elovl5a*-WT construct. Gel was run at 70V for 45 minutes. Bands were compared to the 1kb GeneRuler DNA ladder. **B:** *elovl5b*-WT-S and *elovl5b*-WT-L colony PCR products. Ten colonies were tested per product. No visible bands to indicate successful transformation with the *elovl5b*-WT constructs. Gel was run at 70C for 45 minutes and the bands were compared to 1kb GeneRuler DNA ladder.

Before ligation of WT and synthetic promoter sequences into the pGL4.10[luc2] expression vector, a series of enzyme digestion reactions were performed for quality control and preparation for ligation reactions. All digestion reactions were verified by product size (**Figure 22**). Some colonies formed on the negative ligation control dish, but

very few compared to the positive reactions. Control digestions were then performed to verify that the previously promoter-less expression vector now contained the promoter constructs (**Figure 23 A**). All but one ligation reaction were successful; the synthetic *elovl5a* promoter construct in pGL4.10[luc2]. A second ligation reaction was run for this reaction only, and a new control digestion was run, confirming that the ligation was successful (**Figure 23 B**). This time dephosphorylation of the linearized vector was performed in advance to ensure that the vector did not re-circularize. No colonies were observed on the negative ligation control dish that time.

After cloning into the pGL4.10[luc2] expression vector, the *elovl5*-WT and synthetic promoter contructs were verified by Sanger sequencing (Eurofins Genomics, Moss, Norway).

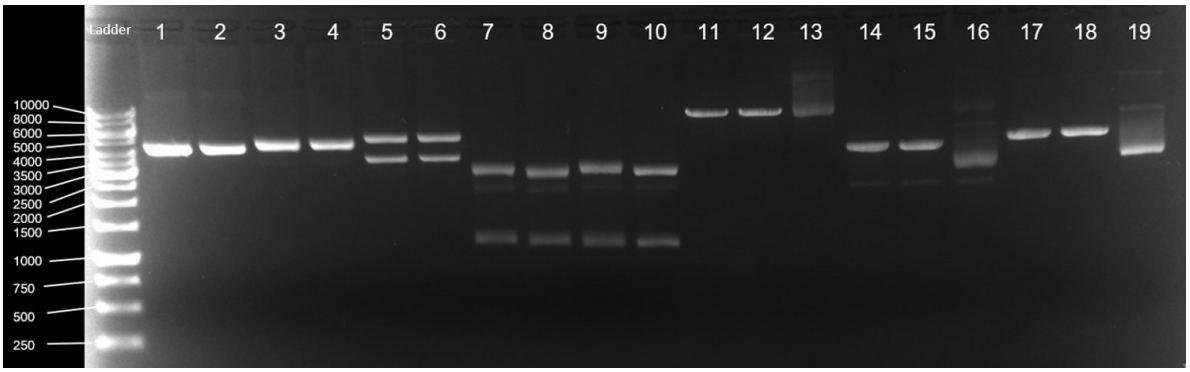


Figure 22. Digested samples run on 1.0% agarose gel. The expression vector (pGL4.10[luc2]) was linearized, using two pairs of restriction enzymes, XhoI and SacI-HF (**columns 1-2**) and HindIII-HF and KpnI-HF (**columns 3-4**). Linearized pGL4.10[luc2] should be 4242 bp in length. The elovl5a WT promoter insert (4913 bp) was cut from the pCR-BluntII-TOPO vector (3519 bp) by restrictions enzymes SacI-HF and XhoI (columns 5-6). The synthetic elovl5a and b ATAC-seq peak inserts were cut from the pUC57 plasmid using the restrinction enzymes pair HindIII-HF and KpnI-HF (**columns 7-8 and 9-10**, respectively). Wells 11-19 are controls. Columns 11-13 are digestion of the topo-elovl5a(wt) vector with SacI only, XhoI-HF only, and no enzymes. **Columns 14-16** are digestion of the pUC57-peaks-elovl5a vector with HindIII-HF only, KpnI-HF only, and no enzymes. **Columns 17-19** were digestions of the pUC57-peaks-elovl5b, set up like 14-16. Bands were compared to the 1kb GeneRuler DNA ladder. Gel was run at 70V for 1 hour.

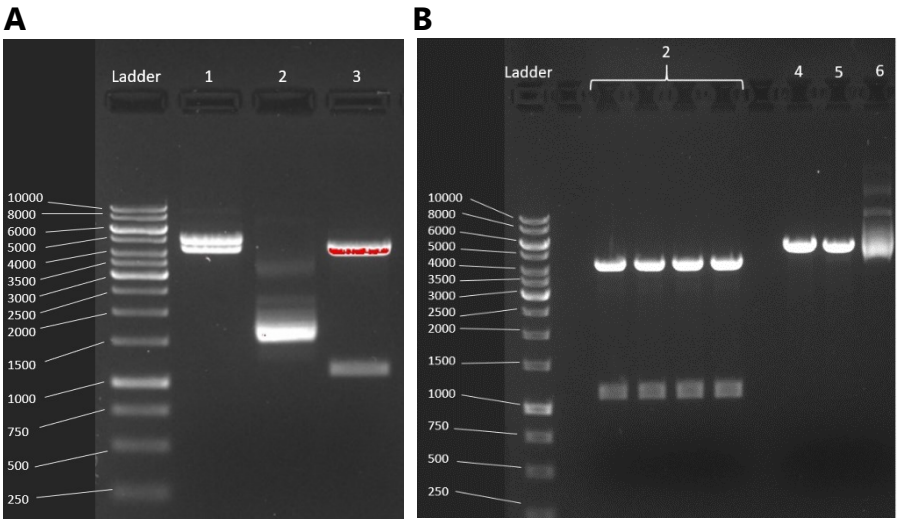


Figure 23. Control digestion of plasmids for promoter reporter assay. **A:** Control digestion of pGL4.10[luc2]-elovl5a(wt) (1), pGL4.10[luc2]-peaks-elovl5a (2) and pGL4.10[luc2]-peaks-elovl5b (3). **B:** A second control digestion of the pGL4.10[luc2]-peaks-elovl5a (2). Controls; With HindIII-HF only, KpnI-HF only and no enzymes (4-6). The pGL4.10[luc2] backbone should give a band at 4242 bp, the elovl5a WT insert at 4913 bp, the elovl5a-peaks insert at 1154 bp and the elovl5b-peaks insert at 1083 bp. The gel was run at 70V for 1 hour. Bands were compared to the 1kb GeneRuler DNA ladder.

3.6.2 Reporter expression in transfected cells

3.6.2.1 RFP expression

RFP was used as positive control for transfection in the promoter reporter experiment. Fluorescent microscopy images of the RFP expressing cells (**Figure 24**) exhibit a greater frequency of RFP expressing cells compared to any of the previous transfections. The RFP expressing cells are clearly clustered together, reflecting the cell structure formation.

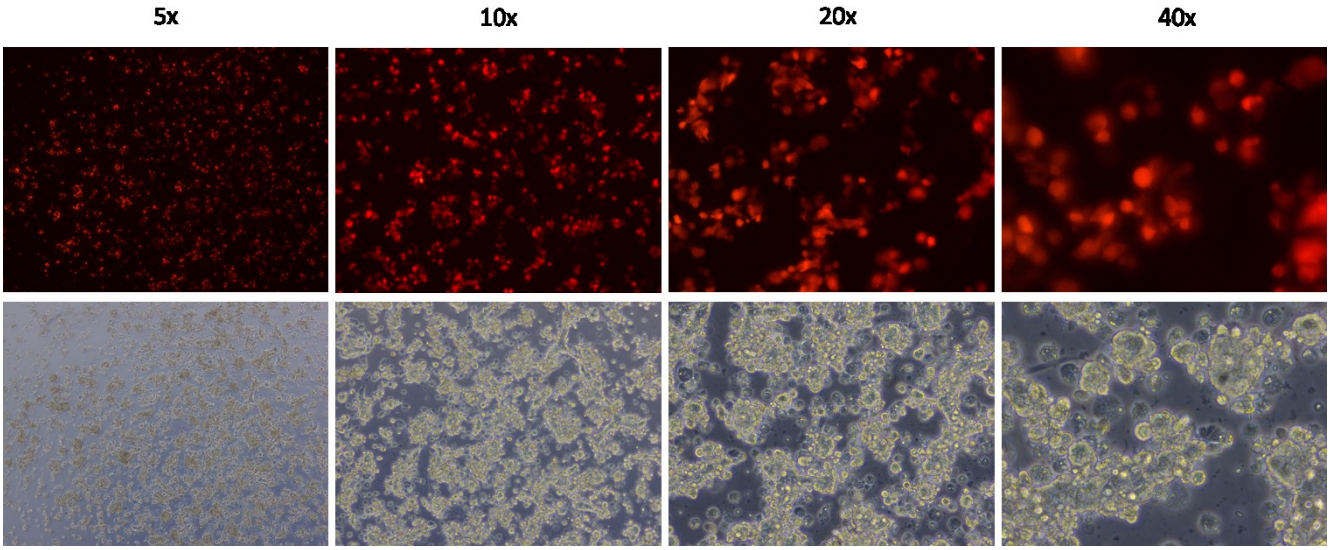


Figure 24. RFP expression for promoter reporter assay experiment – positive control. RFP expressing cells 48 hours post transfection at 5x, 10x, 20x and 40x.

3.6.2.2 Dual-Glo Luciferase Assay

The cells transfected with WT and synthetic promoter consctructs were co-transfected with a renilla luciferase vector and subject to a Dual-Glo Luciferase Assay. No significant cell loss was observed after changing the medium. The lysis reaction was controlled 10 minutes after addition of the Dual-Glo Luciferase Reagent and all cells seemed to be lysed.

The luciferase activity readings show two clear patterns. Firstly, the synthetic *elovl5a* promoter driven luciferase expression is significantly higher than the elovl5a-WT promotor driven expression, with a t-test p-value of 0,006091 (**Figure 25**). Secondly, the synthetic *elovl5b* promoter does not induce transcription in primary liver cells (**Figure 25**).

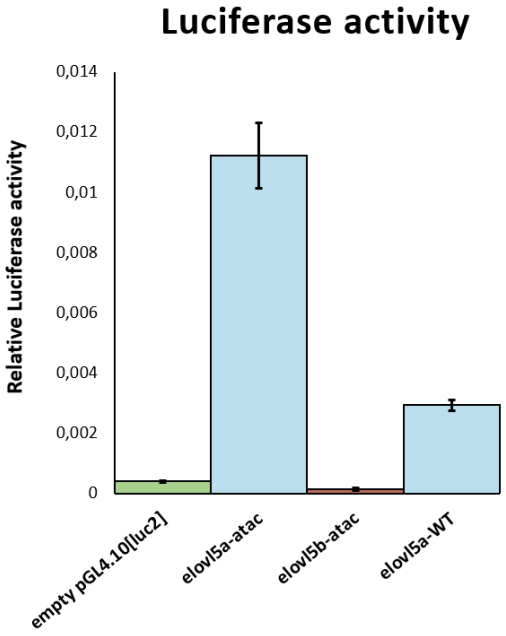


Figure 25. Dual-Glo Luciferase Assay readings. Renilla and firefly luciferase activity was measured using a BioTek Synergy H1M plate reader. Bars show standard deviations. One replicate reading (*elovl5a*-WT, replicate 3) is omitted from the data due to a pipetting error.

4 Discussion

Primary cells are notoriously difficult to transfect. Working with primary cultures is often labor-intensive and protocol optimization often proves necessary. The primary aim of this thesis has been to optimize transfection protocol for primary hepatocytes from Atlantic salmon, and thereby providing a biologically relevant study system for functional studies into Salmonid gene expression regulation. The secondary aim was to use the optimized transfection protocol to study the cis-regulatory elements contributing to transcriptional regulation of the elovl5 duplicate genes in Atlantic salmon using promoter reporter experiments. Although both these genes are expressed in liver, one of them has gained a significantly stronger expression in liver than the other.

This discussion is divided into five parts. Firs, the primary cell isolation is discussed. Then, the use of different transfection assays on primary hepatocyte cells in general, transfection optimization results, and what is left to explore regarding additional factors that can influence the transfection results. Finally, the results from the *elovl5* promoter reporter comparison are scrutinized, and future avenues to improve our understanding of tissue expression divergence among gene duplicates in general are suggested.

4.1 Primary cell isolation is a critical step in transfection protocols.

The results of the perfusion were variable. Throughout this study several smaller modifications were made to the primary cell isolation protocol. However, the impact of these modifications was not assessed using rigorous experimental designs as the primary cell isolation was not within the scope of this project. Nevertheless, our results seem to suggest that viability and transfection efficiency were correlated, hinting towards the importance of healthy cells as a starting point for transfection.

One variable that likely impacted the quality of primary cell culture was the perfusion step. Unfortunately, it proved very hard to achieve consistent perfusion results. A main reason for this was difficulty in locating the portal vein. Other variables like size and shape of the liver made every fish slightly different, and smaller fish seemed more difficult to perfuse than larger fish. Further studies might benefit from using livers of more homogenous size to reduce experiment-to-experiment variation in cell culture quality.

Another variable that seemed to impact primary cell cultures was the collagenase activity in the collagenase perfusion buffer. After we improved this activity (see section 2.4.3.1), we observed less cell agglomeration, which likely both reduced the problem of cells getting stuck in the ECM and influenced cell viability as less pipetting was needed. It should be mentioned that cell damage and subsequent DNA leakage is also likely to cause agglomeration and could be the result of several things, like a long perfusion, exposure to high temperatures, too high centrifugation speed.

4.2 Chemical transfection was inefficient in primary hepatocytes

Testing different transfection methods clearly demonstrated that cationic lipied mediated transfection was extremely inefficient (<<1% cells transfected, **Figure 15**, supplementary table 8). There is no consensus as to why lipid-based chemical transfection occasionally fails in certain cell types. However, this technique depends on integration of lipoplexes through endocytocis, DNA release and subsequent nuclear entry, and is thought to be more efficient in rapidly dividing cells due to the breakdown of the nuclear envelope (Escriou et al., 2001; Mortimer et al., 1999; Riddle et al., 2007). The innate immune response system of the cells is also thought to play a part in lipid-based transfection (Audouy & Hoekstra, 2001). It could be that the innate immune responses of primary cells are more efficient due to exposure to selection pressure and that this might inhibit uptake of foreign DNA. An additional factor in this case might be the transfection temperature. Successful chemical transfection in mammals, but also in fish, are usually performed at temperatures higher than 15°C (Marivin et al., 2015). It might be that the temperature of 15°C used in our study is inhibiting lipoplex movement across the cell membrane (Marivin et al., 2015).

Despite the low chemical transfection efficiencies, we did see significant differences in transfection results between optimization experiments. Generally, Lipofectamine 3000 performed better than Lipofectamine LTX (**Figure 15 B**), extended recovery time (48 hours, **Figure 15 C**), and we find some evidence to support that incubation/transfection temperature is important (**Figure 15 C**), in line with findings in Marivin et. al (2005).

In addition to the factors used to optimize chemical transfection, we could also have optimized lipoplex formation time, tested different lipofectamine:DNA ratios or other types of lipid-based transfection reagents. However, with the extremely inefficient transfection efficiencies we observed, we decided to move to the more promising electroporation technique.

4.3 Optimization of electroporation transfection

Compared to chemical transfection, electroporation, electroporation produced very high transfection efficiencies, up to 30% (**Figure 16, 17, 18, 19** and **24**). These results corresponds with previous findings, which state that electroporation can be an efficient plasmid delivery method in hard-to-transfect cells (Gao et al., 2012; Marivin et al., 2015; Maurisse et al., 2010).

Of the electroporation programs that were tried, 5, R and 16 produced the highest transfection efficiencies (**Figure 17** and **19**), and two of the same programs (16 and R) also performed well in a recent study transfecting SHK-1 cells with RNPs (Gratacap et al., 2020). We also observed some systematic effects on transfection efficiency (**Figure 17**), with higher volts (e.g. 5, 9, 12 and 16) generally resulting in better transfections when comparing with low volts (e.g. 6, 10, 17, 18 and 19). A similar pattern was found in a previously published transfection study in primary granulosa cells from rainbow trout

(Marivin et al., 2015). In addition, the transfection efficiencies tended to be higher using intermediate pulse widths (20-30ms).

Finally, two of the parameters of our best performing transfection program (20ms and 2 pulses, program 16), also produced best transfections in Chinook salmon (Collet & Lester, 2011). Otherwise, program 16 is quite different from the best performing transfection programs of other comparable fish cell transfecion studies in rainbow trout and Chinook salmon (Chi et al., 2011; Marivin et al., 2015). These differences could be due to variations in factors like cell type, plasmid size and electroporation buffers, and highlights the importance of optimization of transfection protocols.

4.4 Transfection efficiency estimation

The major aim of our transfection experiments is to achieve high enough proportions of transfected cells to conduct promoter reporter assays with good sensitivity. If there are too many untransfected relative to transfected cells, it will hamper our ability to detect small, but biologically meaningful differences in promoter/enhancer function.

A key challenge in this work has therefore been to produce high and reproducible transfection efficiencies. Our use of different methods for transfection efficiency estimation between experiments are therefore not optimal and makes direct cross-experiment comparisons difficult. Futhermore, each efficiency estimation method comes with its own inherent challenges.

In cases where it was not possible to quantify transfection efficiency from cell counts and flow cytometry, efficiencies were estimated and compared by visual inspection of samples under a fluorescent microscope. This approach is vulnurable to subjective bias, variation in cell number between conditions, and variation in transfection efficiency between samples.

In many experiments, a hemocytometer was used to count the number of live cells as well as RFP expressing cells. Since primary hepatocytes adhere to the surface of culture wells, trypsination is necessary prior to the use of a hemocytometer. Following trypsination, cells are pelleted and resuspended. Occasionally, the small cell pellets that formed, or parts of them, were lost in this process, which affected counting of live cells. Loss of cells could result in inaccurate transfection efficiencies or inability to get cell counts at all. In addition, the cells tended to cluster together, which could also have impacted the accuracy of hemocytometer.

In one experiment we used flow cytometry to estimate transfection efficiency. This method has been has been praised for its high sensitivity (Ducat et al., 2011; Homann et al., 2017), but it has also been critizised for being slightly subjective in nature, in that both gating of cell population, and subsequent identification of positive cells, is done manually by visual inspection of light scatter plots and light intensity histograms (Aghaeepour et al., 2013). If the gate is too wide there are risks of including background noise, like cell debris and other cell types, which decreases accuracy.

In conclusion, despite all the uncertainty linked to the absolute estimates of transfection efficiencies, we are confident that the general trends regarding transfection efficiency holds up.

4.5 Elovl5 promoters induce expression in primary hepatocytes

The reporter assays using *elovl5* promoters reveal two interesting results. Firstly, we can show that our primary hepatocyte transfection protocol is suitable for further in-depth mechanistic studies on liver-centric genes. This could enable the research community to move away from studying promoter function in cells of unrelated species or tissues, which has been a weakness in previous studies on salmon liver gene regulation (Carmona-Antoñanzas et al., 2016; Leaver et al., 2014). Secondly, we found unexpected regulatory effects of the *elovl5* synthetic promoter constructs.

Contrary to expectations, the *elovl5a* synthetic promoter produced higher luciferase expression than the *elovl5b* synthetic promoter. Not just that, but the *elovl5b* synthetic promoter driven expression was below that of the empty vector (no promoter). There might be two possible explanations for this, one is that technical error(s) have occured during the experiment. Such errors might be that plasmid have been omitted from the sample or the samples were not electroporated. The other is biological. The synthetic promoters used in this experiment contained the transcription factor binding sites suspected to drive the divergent regulation (Carmona-Antoñanzas et al., 2016) in the duplicates. From the results presented here, it is possible that there are other contributing binding sites outside of the promoter region. It could be that sequence divergence within the intronic regions of the *elovl5* duplicates might be significant to the observed *elovl5* expression divergence in Atlantic salmon. Intronic regions have been known to affect transcription levels by harboring enhancer elements (Wei et al., 2006), but also through intron mediated enhancement (IME), wich is a collective term for mechanisms that enhance gene expression without involving binding of transcription factors (Chorev & Carmel, 2012; Shaul, 2017). However, this type of expression elevation only occurs if the intronic sequence is present in the transcribed region. The Carmona-Antonanzas et. al. study (2016), present the possibility that the TE insertion events that occured after the Salmonid WGD could have contributed novel CREs to these regions. There could also be other explanations, like a significant distal enhancer further upstream that is omitted from our constructs (Schoenfelder & Fraser, 2019), regulatory effects of flanking genes (Gherman et al., 2009) or it could be that the ATAC-peaks simply do not represent all sequences bound by transcription factors. Still, it is very hard to explain why the synthetic *elovlb*-promoter did not yield any LUC signal at all with biological causes.

Interestingly, the observed *elovl5a*-WT driven luciferase expression was below that of the *elovl5a* synthetic promoter. It might be that the synthetic promoter is a more efficient/stronger promoter than the WT variant. By actively removing parts of the regulatory sequence, repressor- and co-repressor activity could potentially be inhibited somehow. This, as well as sequence shuffles in themselves, could also have affected the assembly of the transciptional machinary, which might occur more efficiently.

In addition, previous studies have found that the spacing between transcription factor binding sites affects transcriptional levels in higher order eukaroytes (Zhang et al., 2008).

Initially, the intention was to compare the *elovl5a*-WT driven expression with that of the *elovl5b*-WT, but the *E. coli* transformation with the elovl5-WT promoter failed. This was likely the result of low DNA concentration. The *elovl5b* contruct had a high GC- and repeat content, which made it difficult both to synthesize the synthetic promoter and to amplify the WT by PCR. These sequence traits pose some challenges because they can cause formation of *s*econdary structures as well as mispriming. It wasn't before DMSO was added to the PCR reaction that PCR yielded some results. DMSO can inhibit secondary structure formations in the DNA template or in the primers themselves (Strien et al., 2013). However, the *elovl5b*-WT PCR gave multiple bands, both longer and shorter than the construct itself. This could be due to sequence repeats enabling primers to bind at other sites. Thus, DNA for *E. coli* transformation had to be extracted from the gel, which produced a rather low DNA concentration. After transformation with the two possible *elovl5b*-WT amplicons, only a limited number of transformants were observed (supplementary table 9). In the end, colony PCR revealed that none of these contained the correct inserts. The PCR could perhaps be optimized by reducing the primer concentration, thereby decreasing the probability of the primers binding to non-target-regions and also avoid formation of primer-dimers.

5 Conclusion

Immortalized cell lines are often used for functional studies, since they can be easily accessed and often have good protocols for relatively efficient transfection. Yet, performing functional studies in primary cells from biologically relevant tissues potentially yield better biological insights. The optimized protocol for transfection of Atlantic salmon primary hepatocytes that is presented here hopefully opens possibilities for further studies of Salmonid gene expression regulation in liver.

In this work we show that electroporation is the better option for transfection in primary hepatocytes. Even though there are drawbacks to electroporation in the form of cytotoxic effects, we demonstrate sufficient transfection efficiencies for reporter gene experiments. Further investigation of the cytotoxic effects of electroporation should still be considered to potentially identify effects that might interfere with experimental signals.

The results from the final promoter reporter assay deviated radically from our expectations, with the hypothesized strongest liver promoter displaying almost no induction of transcription. These experiments must therefore be repeated independently, before we can draw any conclusions. Nevertheless, the *elovl5* experiment can be consideres a success as an initial 'proof of principle' for our primary cell transfection protocol for future functional studies.

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Appendix

Supplementary table 1. Transfection reagent details for chemical transfection optimization. See section 2.4.1.2)

Volumes per well (24-well plate format)					
		Lipofectamine 3000		Lipofectamine LTX	
		High	Low	High	Low
Step 1 L-15 + Lipo	L-15 (ren)	25 µl	25 µl	50 µl	50 µl
	Lipofectamine 3000/Lipofectamine LTX	1.5 µl	0.75 µl	5 µl	2 µl
		Vortex 2-3 sek			
Step 2 L-15 + DNA + P3000 Reagent	Opti-MEM	50 µl	50 µl	250 µl	250 µl
	Plasmid DNA	1 µg	1 µg	5 µg	5 µg
	P3000/Plus Reagent	2 µl	2 µl	5 µl	5 µl
		All of the DNA-dilution added to the Lipo-mix			
		Incubation for 15 min. at RT			
		Added dropwise to cells			

Supplementary table 2. A 24-well electroporation optimization with the Neon Transfection System. Programs tested in optimization III (section 2.4.3.2).

Sample	Voltage	Pulse width	Pulse no.
1	Un-electroporated sample		
2	1400	20	1
3	1500	20	1
4	1600	20	1
5	1700	20	1
6	1100	30	1
7	1200	30	1
8	1300	30	1
9	1400	30	1
10	1000	40	1
11	1100	40	1
12	1200	40	1
13	1100	20	2
14	1200	20	2
15	1300	20	2
16	1400	20	2
17	850	30	2
18	950	30	2
19	1050	30	2
20	1150	30	2
21	1300	10	3
22	1400	10	3
23	1500	10	3
24	1600	10	3

Supplementary table 3. Electroporation transfection details. *Note the difference between live cells/well and cells/well.

	Transfection optimization II	Transfection optimization III	Transfection optimization IV	Promoter reporter assay	
Plate format	24-well plate			6-well plate	24-well plate
Culture medium (mL)	0.5			2	0.5
Cells/well	100 000 live* cells/well	100 000 cells/well	100 000 cells/well	100 000 live* cells/well	400 000 live* cells/well
Neon tips (µl)	10				
Plasmid (µg)	1			4	1

Supplementary table 4. PCR primers. Primer sequences used to amplify the elovl5a WT promoter region from Atlantic Salmon gDNA during PCR.

Construct	Forward primer (5'→ 3')	Reverse primer (5'→3')
elovl5a-WT	AATGAGCTCAGCTCTGCAAAGCCATGTG	AATCTCGAGTTCTGACCTAAATAGACAGATG

Supplementary table 5. Colony PCR primers. Primer sequences used for colony PCR to verify the presence of an insert of correct size in the pCR™-BluntII-TOPO vector

Insert	M13 Forward primer (5'→ 3')	M13 Reverse primer (5'→3')
elovl5a-WT	GTAAAACGACGGCCAG	CAGGAAACAGCTATGAC
elovl5b-WT	GTAAAACGACGGCCAG	CAGGAAACAGCTATGAC

Supplementary table 6. Sanger sequencing primers. Primer sequences used to for Sanger sequencing performed by Eurofins (Moss, Norway) for sequence verification

Construct	Forward primer 1 (5'→ 3')	Forward primer 2 (5'→ 3')	Reverse primer (5'→3')
elovl5a-WT	TATTGGACACGTCTCTCTGTGG	-	ACCTATGTGCAATGCAAGGAAG
Synthetic *elovl5a*	AGTGCAAGTGCAGGTGCCAG	GGTGCGGCATAACCAATCAG	-
Synthetic *elovl5b*	AGTGCAAGTGCAGGTGCCAG	ATACACGTACCGCGTTCAAC	-

Supplementary table 7. Quantitative cell culture data. Fish sex, weight and length, in addition to cell count and viability of the single cell suspension. Note that Automatic cell counter was used to get cell counts in optimization I. Hemocytometer was used for the rest of the experiments. *The number used to calculate amount (µl) of cell suspension needed for the experiment.

	Transfection optimization I	**Transfection optimization II**	**Transfection optimization III**	**Transfection optimization IV**	**Transfection for promoter reporter assay**
Transfection method	Chemical transfection	Electroporation	Electroporation	Electroporation	Electroporation
Quantitative cell culture data					
Fish sex	Male	Male	Male	Male	Male
Fish weight (g)	493	120	236	179,4	171.6
Fish length (cm)	33	22	27	26	25,5
Total cell count (cells/mL)	$15{,}3 \cdot 10^6$ *	$7{,}88 \cdot 10^5$	$29{,}2 \cdot 10^6$ *	$25{,}8 \cdot 10^6$ *	$24{,}2 \cdot 10^6$
Live cell count (cells/mL)	$12{,}09 \cdot 10^6$	$6{,}3 \cdot 10^5$ *	$19{,}86 \cdot 10^6$	$21{,}9 \cdot 10^6$	$21{,}8 \cdot 10^6$
Cell viability (%)	79%	80%	68%	85%	90%

Supplementary table 8. Estimated transfection efficiencies after chemical transfection. Transfection efficiency was estimated by: (RFP expressing cells/seeded cells) x viability. In both *20°C incubation* samples, transfection efficiency was measured using fluorescent microscope, Trypan blue staining and hemocytometer (fraction of RFP expressing cells of live cells). *No data. See section 2.4.1.2.

RFP count data, estimated transfection efficiency (TE) and relative light unit (RLU)							
Reporter gene	Incubation temperature	Time of tranfection	Transfection reagent	Ratio	Replicate	No. of fluorescent cells after 4/5 days	Estimated TE (transfection efficiency (%))
RFP	15°C	Reverse transfection	Lipofectamine 3000	High	1	70	0.01842105
					2	100	0.02631579
				Low	1	5	0.00131579
					2	15	0.00394737
			Lipofectamine LTX	High	1	0	0.00
					2	1	0.00026316
				Low	1	0	0.00
					2	0	0.00
		24 h	Lipofectamine 3000	High	1	30	0.00789474
					2	50	0.01315789
				Low	1	*	*
					2	*	*
			Lipofectamine LTX	High	1	6	0.00157895
					2	5	0.00131579
				Low	1	*	*
					2	*	*
		48 h	Lipofectamine 3000	High	1	150	0.03947368
					2	200	0.05263158
				Low	1	20	0.00526316
					2	70	0.01842105
			Lipofectamine LTX	High	1	6	0.00157895
					2	10	0.00263158
				Low	1	0	0.00
					2	1	0.00026316
	20°C	24 h	Lipofectamine 3000	High	1	10	0.23
					2	*	*
		48 h			1	21	0.39
					2	*	*

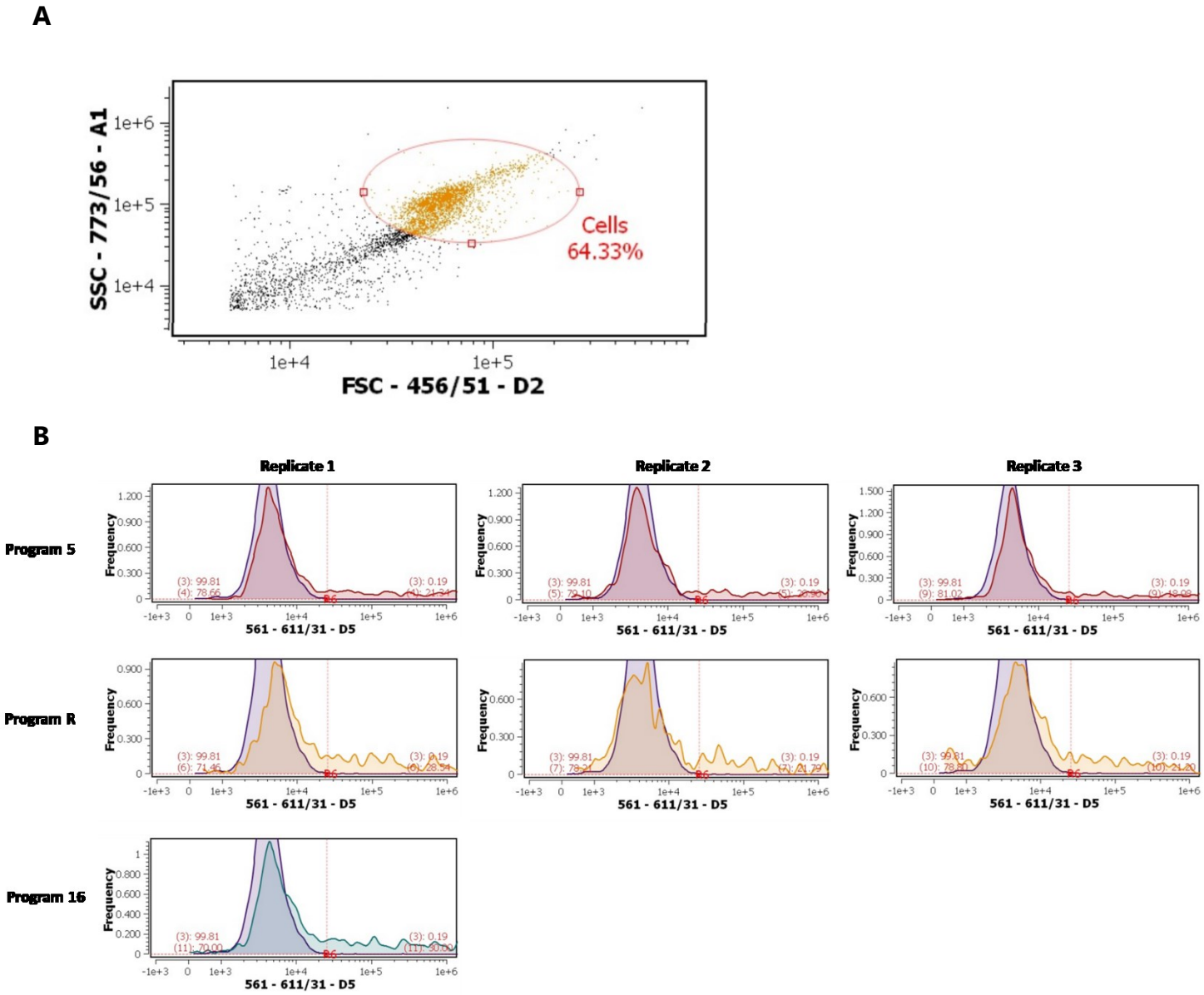

Figure 1. Flow cytometry data analysis to measure transfection efficiency. A: Identification and gating of the hepatocyte cell population. Light scatter data from an un-transfected sample was used to identify the target cell population by size. Cells of the same size have clustered together. **B**: Identification of RFP expressing cells. Intensity detected in the 611 nm channel plotted against number of events detected at that intensity, for all replicates from all electroporation programs. Positive sample data histograms was overlaid a negative sample data histogram (purple peak). The red, orange and green peaks are the electroporated samples capturing red fluorescent protein emisson at 611 nm. Two replicates from electroporation program 16 are missing.

Supplementary table 9. Number of colonies after transformation of competent OneShot TOP10 *E. coli* cells with pCR-BluntII-TOPO vector containing the promoter constructs, using the Zero Blunt TOPO PCR Cloning kit (ThermoFisher, 2014).

	elovl5a-WT	*elovl5b*-WT-S	*elovl5b*-WT-L
50 ul plate	63	7	1
250 ul plate	228	15	10

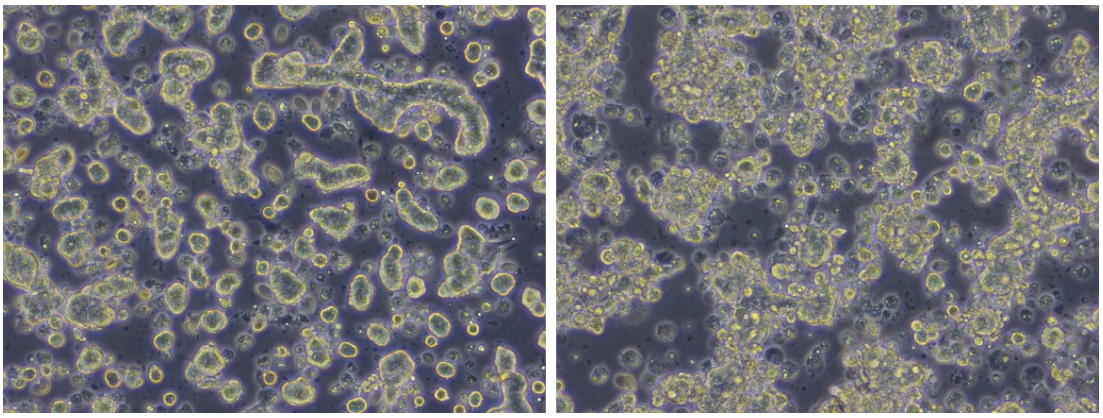

Figure 2. Un-electroporated and electroporated sample after electroporation for the reporter assay. Cells transfected with RFP were compared to un-electroporated cells after 48 hours. The un-electroporated sample displayed considerably more structure formation than the electroporated sample.

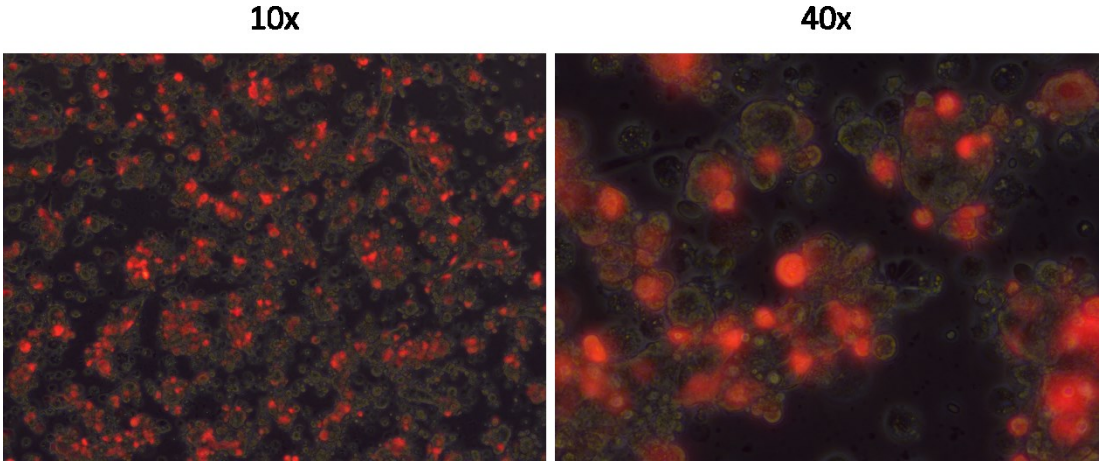

Figure 3. Image overlay of RFP expressing cells after electroporation for the reporter assay.

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