DEPARTAMENT OF
LIFE SCIENCES

NOVA SCHOOL OF
SCIENCE & TECHNOLOGY

INES FERREIRA DE ALMEIDA
BSc in Biochemistry

Optimization of *in vivo* electroporation and comparison to microinjection as delivery methods for transgenesis in zebrafish (*Danio rerio*). Generation of a new neuronal zebrafish line.

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE
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In memory of grandma Conceição and grandpa Armindo,
you will forever be missed.

ACKNOWLEDGMENTS

What an incredible journey this year has been. It has made me grow professionally and personally. This project is now coming to an end, and I would like to show my appreciation to all the people who somehow contributed to it.

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"The greatest enemy of knowledge is not ignorance; it is the illusion of knowledge."

(Stephen Hawking)

ABSTRACT

Transgenic zebrafish are important models for biomedical research. There are several technologies available for the generation of transgenics and for genome editing. However, methods for the delivery of exogenous components remain limited. In Zebrafish, the most used method is microinjection, which requires sophisticated technical skills and presents a low integration rate of large constructs. Alternatively, a few studies reported the use of electroporation as a delivery method for the generation of transgenic zebrafish; however, these protocols contain some limitations that reduce their widespread applicability. To overcome this, we based on the most recent published work reporting electroporation in zebrafish embryos, to implement optimizations in order to increase the number of embryos electroporated, the efficiency of plasmid DNA delivery and its integration in the germline. Electroporation rounds of 30 one-cell stage zebrafish embryos with 300 ng/uL of plasmid DNA in PBS using 35 V poring pulse and 5 V transfer pulse yielded the highest survival and efficiency. Compared to microinjection, the optimized electroporation protocol achieved similar fluorescence intensity and expression pattern, opening the way to becoming a practical and efficient alternative to microinjection.

In parallel, a new calcium indicator pan-neuronal transgenic zebrafish line, elalv3:GCaMP6fEF05 was generated, through microinjection into one-cell stage zebrafish embryos, followed by 3 rounds of fish crosses, screens, selection and raising. The improvement of delivery methods, such as electroporation, will expand the generation of new zebrafish lines for the study of developmental and molecular biology that ultimately allows the exploration of new human therapeutic avenues.

Keywords: Zebrafish embryos, Transgenesis, Microinjection, Electroporation, Genetically encoded fluorescent reporters

RESUMO

Os peixes-zebra transgénicos são modelos importantes para a pesquisa biomédica. Existem várias tecnologias disponíveis para a geração de transgénicos e edição do genoma. No entanto, os métodos para a entrega de componentes exógenos permanecem limitados. No peixe-zebra, o método mais utilizado é a microinjeção, que requer habilidades técnicas sofisticadas e apresenta taxa de integração de grandes construções reduzida. Alternativamente, alguns estudos relataram o uso de eletroporação como um método de entrega para a geração de peixes-zebra transgénicos; no entanto, esses protocolos contêm algumas limitações que reduzem sua aplicabilidade generalizada. Como tal, tendo por base um trabalho publicado recentemente relatando a eletroporação de embriões de peixe-zebra, implementaram-se otimizações a fim de aumentar o número de embriões eletroporados, a eficiência da entrega de DNA plasmídico e a sua integração na linha germinativa. Ciclos de eletroporação de 30 embriões de peixe-zebra no estado de uma célula com 300 ng / uL de DNA plasmídico em PBS usando um pulso de formação de poros de 35 V e pulso de transferência de 5 V obtiveram a maior taxa de sobrevivência e eficiência. Comparado à microinjeção, o protocolo de eletroporação otimizado alcançou uma intensidade de fluorescência e padrão de expressão semelhantes, abrindo caminho para se tornar uma alternativa prática e eficiente à microinjeção.

Em paralelo, uma nova linha de peixe-zebra transgénica pan-neuronal, elalv3: GCaMP6fEF05 foi gerada, através da microinjeção em embriões no estado de uma célula, seguida por 3 rondas de cruzamentos de peixes, *screens*, seleção e criação. A otimização dos métodos de entrega, como a eletroporação, permite expandir a geração de novas linhas de peixe-zebra para o estudo da biologia molecular e do desenvolvimento que, em última análise, permite a exploração de novos caminhos terapêuticos para humanos.

Palavras chave: Embriões de peixe-zebra, Transgénese, Microinjeção, Eletroporação, Repórteres fluorescentes geneticamente modificados

CONTENTS

LIST OF FIGURES

List of Tables

GLOSSARY

Alternating current	Oscillating dielectrophoretic current in which an electrical current rises to a maximum point in one direction and falls to zero and then rises in the opposite direction and then repeats
Anode	Positive electrode or terminal of a device from which electrons flow outwards
Capacitance	Quantity of electric charge which a capacitor can receive with an applied voltage, measured in Farads
Cathode	Negative electrode or terminal of a device to which electrons flow towards
Conductance	Measure of how easily electrical current can pass through a material, measured in Siemens
Current	Rate at which electric charge flows past a point in a circuit, measured in Amperes
Pulse Length	Length of time the cell is exposed to the electrical field, usually measured in milliseconds
Resistance	Opposition to current flow and dissipation of energy in the form of heat, measured in Ohms
Square wave	A wave form that alternates between two fixed values for an equal amount of time
Transposon	Repetitive DNA sequences that move from one location on the genome to another

Transgenic Deliberately introduction of one or more foreign DNA sequences by artificial means into the genome

Voltage Potential difference in charge between two points in an electrical field, measured in Volts

ABBREVIATIONS

ANOVA	Analysis of variance
CaM	Calmodulin
CLS	Centralized Life Support
CRISPR	Clustered Regularly Interspaced Palindromic Repeats
DNA	Deoxyribonucleic acid
dpf	Days post fertilization
E3	Embryonic medium
EGFP or GFP	(Enhanced) Green fluorescent protein
GECI	Genetically encoded calcium ion indicators
H2B	Histone 2B
hpf	Hours post fertilization
Isl2b	Islet2b
IVF	*In vitro* fertilization
LSSmOrange	Light-induced Spectral Shift (LSS) Monomeric Orange fluorescent protein
mRNA	Messenger Ribonucleic acid
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
PTU	1-phenyl 2-thiourea

RNA	Ribonucleic acid
STD	Standard deviation
TALENs	Transcription activator-like effector nuclease
TeO	Optic tectum
Tg	Transgenic
TILLING	Targeting Induced Local Lesions in Genomes
Tricaine	Ethyl 3-aminobenzoate methanesulfonate
TU	Tübingen
UAS	Upstream activating sequence
UV	Ultraviolet
ZNFs	Zinc Finger Nucleases
β-DHP	β-Dihydroprogesterone

UNITS

bp	base pair
g/L	gram per liter
Kb	kilobase
KΩ	kiloohm
L	liter
mg/mL	milligram per milliliter
mL	milliliter
mM	millimolar
mm	millimeter
ms	millisecond
ng/µL	nanogram per microliter
nm	nanometer
nM	nanomolar
° C	degrees
ppm	parts-per-million (10^{-6})
V	volts
w/v	weight per volume (mass concentration)
µL	microliter
µS	microsiemens

1

INTRODUCTION

Understanding the pathophysiological molecular mechanisms of human diseases and finding new possible therapeutic approaches requires the right disease models and molecular tools [1]–[4]. Certain aspects of disease biology, such as tissue homeostasis, microenvironment, and response to drugs, cannot be fully recapitulated *in vitro*. That is why reliable *in vivo* animal models are so relevant. Through transgenesis, mutations or gene editing, they are capable of mimicking human diseases [5]. The zebrafish is particularly well suited for these applications and is nowadays one of the favourite animal models in biomedical research [1], [6]–[8]. However, to continuously generate transgenic and mutant zebrafish lines that help respond more complex scientific questions, it is important to keep improving and developing new genetic approaches and delivery methods for introducing exogenous molecules into the animals.

1.1 Zebrafish as a model organism

Danio rerio, commonly known as zebrafish, is a small tropical freshwater fish that belongs to the Teleostei infraclass and Cyprinidae family (Figure 1.1). Zebrafish are found in shallow, slow-moving waterways such as small streams, silt-bottom well-vegetated pools, and rice paddies throughout Southern Asia, including India, Bangladesh, and Nepal. The zebrafish is omnivorous, its natural diet consists primarily of zooplankton and insects, although also feeds from inorganic material [9]–[13].

Figure 1.1- Adult Zebrafish. On the top, a female zebrafish, and on the bottom, a male zebrafish. Retrieved from [14].

Traditionally, the zebrafish has been widely used as a model organism in the fields of developmental genetics, vertebrate biology and biomedicine [2], [15]. It has, nevertheless, emerged as an excellent model organism for studies in neuroscience research. [1], [2].

Although phylogenetically distant, zebrafish presents a physiological and anatomical homology to humans, evidenced in most of the organs, including a nervous system organized like in all vertebrates. Zebrafish shares a high genetic similarity to humans, its genome has approximately 70% of homology with the human protein coding genes (Figure 1.2), and 82% of orthologous human disease-related genes. This suggests that most human physiology and pathologies can be modelled in zebrafish [2], [5], [6], [12].

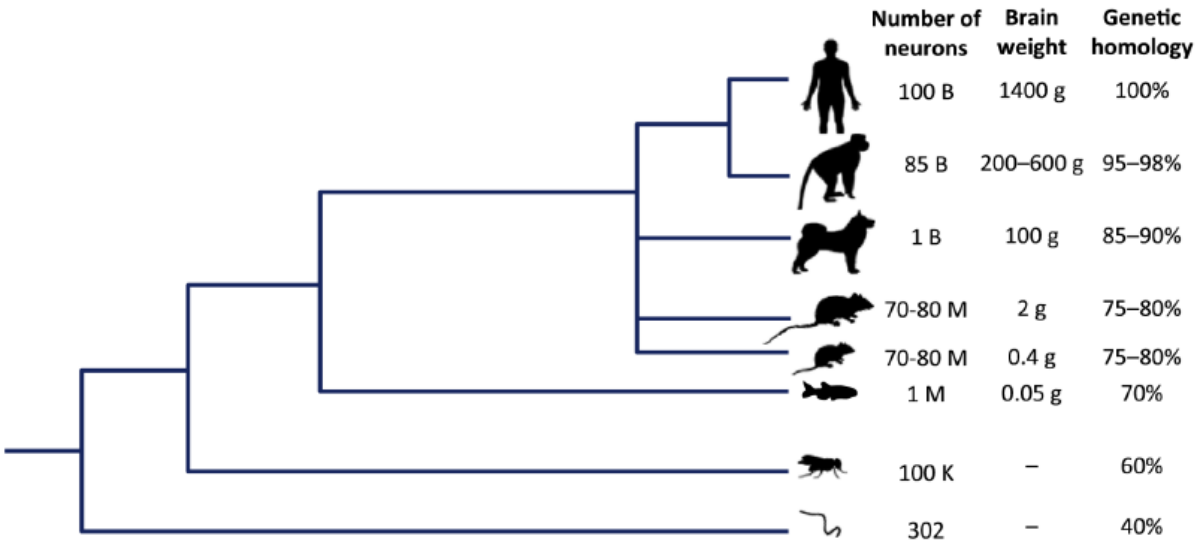

Figure 1.2- Phylogenetical tree of major model species. Comparison of genetic homology, brain weight and number of neurons between the human, monkey, dog, rat, mice, zebrafish, fruit fly and nematode. Adapted from [1].

Zebrafish allows the scalability and the application of many of the genetic tools that made *Drosophila* and *C. elegans* such powerful genetic models while being a vertebrate, and sharing a high level of homology with human genome [11], [16]. Besides, it offers several practical advantages. It is relatively cost-efficient, easy to breed, raise and maintain, and can be

housed in large numbers in a limited laboratory space [2], [5], [6]. It exhibits a rapid embryonic development followed by a short generation time of two to three months [10], [12], [17]. A single pair can produce up to some hundreds of offspring per breeding event, meaning that experiments can be performed with high numbers and high-throughput approaches [12], [16]–[18]. Zebrafish embryos are small, optically transparent and have a rapid external development, allowing thorough observations of processes down to a subcellular level in real time, *in vivo*, without the need for invasive approaches and within the field of view of a standard microscope [6], [12], [17][1], [19], [26]. Also, they are robust and accessible for manipulations such as injection, electroporation, or transplantation from immediately after fertilization. These properties make the zebrafish embryos a great model to study vertebrates development, especially because they develop major organ systems, with the basic organ patterning conserved among vertebrates, within 2 days post fertilization (dpf) [7]. Another field of research that benefits from zebrafish embryos size and optical properties is neuroscience. Other advantages for neuroscience research are the fast neuronal development, relatively simple brain structure and low number of neurons compared to mammals [20], [21], [22]. Around 3 dpf, the first social and cognitive behaviour responses appear, such as food hunting, avoiding predators and movement stability [6], [12], [17], [19], [20]. Furthermore, zebrafish larvae can be partially restrained in low-melting agarose, which enables stable imaging of the brain while the eyes and tail move freely, allowing the association between behaviour and neuronal activity [23].

In addition, zebrafish are genetically tractable, allowing the generation of genetically modified fish lines utilizing genetic techniques [2], [5]. Molecular tools for its genetic manipulation include gene or enhancer-trapping [24]; morpholinos for gene knockdown [16], [25], [26]; targeting induced local lesions on genomes (TILLING) [16], [26], [27]; retroviral vectors and transposon systems such as the Tol2 system for integration of foreign DNA [16], [28]; and gene editing using zinc finger nucleases (ZFNs), transcription activator-like effector nuclease (TALENs) system [16], [29], [30] and clustered regularly interspaced short palindromic repeats (CRISPR) technology [31], [32]. The available genetic approaches allow the generation of zebrafish models for human disorders and hallmarks [6], [11], [16], [18], [33], as for example for studying blood, endocrine and metabolic disorders as well as muscular dystrophies and neurodegenerative syndromes, among others [5], [10], [12], [13], [16], [18], [34].

1.2 Transgenesis

Transgenesis is an essential method for investigating genes function in model organisms. It consists of the introduction of a foreign genetic material into the genome of a host organism [28], [35]. Transgenesis in zebrafish is mainly achieved through delivery of DNA by microinjection in one-cell stage embryos. It started with nacked DNA injections, but low integration yields and high silencing levels across several generations required the adaptation and development of new transgenesis methods. Following that, a method exploring viral vectors was developed [36]. Nevertheless, manipulating and modifying viral vectors is laborious, so other high-efficiency transgenesis methodologies, such as the Tol2 transposon system, were discovered and adapted [37], [38]. Combination of the Tol2 transposon system with the Gal4-UAS transactivation system, explained in the following chapters, has been largely used to produce many transgenic zebrafish lines [36]. Transgenic tools allow for *in vivo* labelling and thorough study of expression and function of specific cell types of interest using fluorescent reporters [7].

1.2.1 *Tol2* transposon system

The Tol2 element is an autonomous, class II transposon from the hAT family of transposable elements (TEs). It was identified from the genome of a Japanese freshwater teleost, medaka fish (*Oryzias latipes*) [36]–[42], and it represents the first naturally active TE element identified from a vertebrate genome [37], [40], [43]. Its autonomy relies on the fact that it encodes a thoroughly functional transposase and so it can catalyse its own transposition into another region of the genome [36]–[40], [42]

The Tol2 transposable element sequence (Figure 1.3), including all coding regions, necessary for transposase production, is 4.7 kilobases (kb). However, the 200 basepairs (bp) (left- 5' end) and 150 bp (right- 3' end) DNA minimal cis-sequence, recognized by the transposase, are sufficient for nonautonomous transposition in the presence of transposase enzyme [35]–[40], [42], [43]. This property was the base for the development of gene transfer vectors for generation of transgenic organisms: DNA fragments can be cloned between these minimal cis-sequences (commonly called Tol2 arms) and delivered to a host cell together with transposase, for integration into the host genome using the natural mechanisms of class II TE transposition [28], [36], [38], [42].

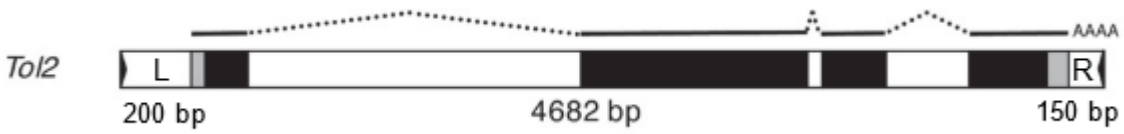

Figure 1.3- Tol2 transposable element. Full-length Tol2 (4682 bp) encodes the transposase gene. Translated regions (black boxes), untranslated regions (grey boxes) and non-coding regions (white boxes). Transcribed RNA is shown by lines and dots illustrating exons and introns, respectively and AAAA tail from polyadenylation. L (200 bp) and R (150 bp) are the minimal Tol2 cis-sequences necessary for transposition. Black arrowheads represent the 12 bp terminal inverted repeats (TIRs) in the minimal Tol2 cis-sequences. Adapted from [43].

More specifically, the tol2 transposition system for transgenesis consists of two components: (i) a transposase mRNA synthesized *in vitro* and (ii) a donor plasmid harbouring a nonautonomous Tol2 construct containing the sequence to be integrated into the host genome flanked by the minimal Tol2 recognition sequences [35]–[40], [44]. The two components are co-injected into the host, which in the case of zebrafish, are typically one-cell stage embryos (Figure 1.4). The transposase is translated from the synthesized mRNA into a functional enzyme that catalyses the excision of the nonautonomous Tol2 construct from the donor plasmid. [36]–[39], [44]. The excised Tol2 construct integrates randomly into the host genome through a cut-and-paste mechanism [35]–[38], [42]. Upon transposase mRNA and protein gradual degradation, the transposase activity ceases and Tol2 insertions become stable [36]–[38], [44]. When the construct integrates in the germline precursor cells, it is transmitted to the progeny, allowing the establishment of stable transgenic fish lines [35], [36].

Tol2 system has been shown to be active in all vertebrates cells tested to date, including fish, frogs, chickens, and mammals cells [36], [38]. In zebrafish, it is highly efficient, with 50-70 % of injected fish being germline-transmitting founders [7], [28], [35]–[38], [40], [44]. Due to this, it became a standard and powerful method in zebrafish [28], [35]–[38]. Its applications include the generation of stable transgenic lines for the study of expression and activity of promoters, enhancers and/or genes of interest [7], [28].

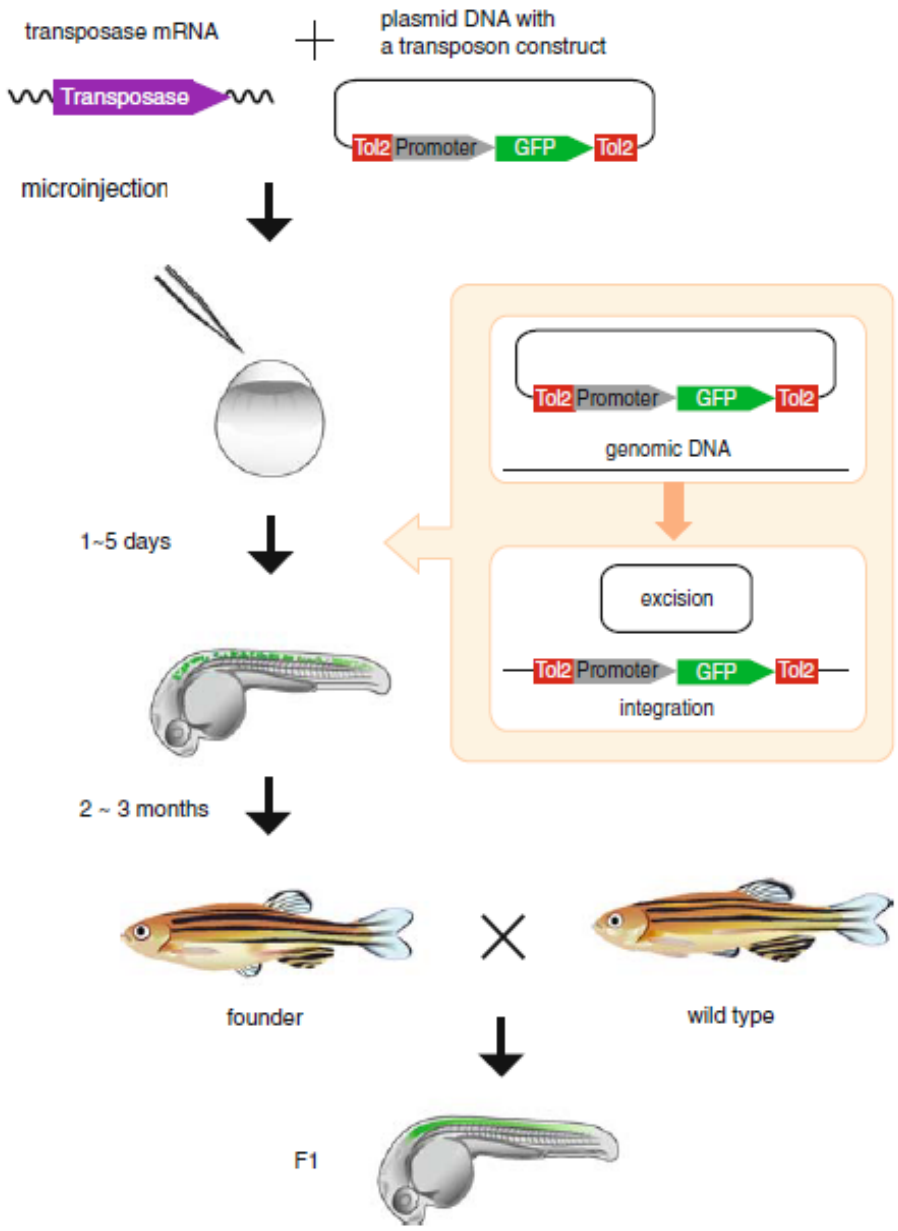

Figure 1.4- Tol2-mediated transgenesis in zebrafish. Transposase mRNA and Tol2 donor-plasmid containing an insert with promoter and a GFP (green fluorescent protein) reporter are co-injected into zebrafish embryos. The Tol2 construct is excised from the plasmid and integrates the zebrafish genome. Injected embryos are raised into adulthood and crossed with wild-type fish. The Tol2 construct integrated in germline cells is transmitted to the offspring (F1 generation). Adapted from [7].

1.2.2 Gal4/UAS transactivation system

The Gal4/UAS transactivation system is naturally present in yeasts, regulating genes implicated in the galactose metabolism [45]. The Gal4 protein is a transcriptional factor [8] that only becomes active upon binding of the specific recognition sequence, the Upstream Activating Sequence (UAS). When that happens, it initiates transcription of the gene

downstream of the UAS, by recruiting co-activators and the transcriptional machinery to the promoter site [46]–[50]. This way, the expression of any given gene located downstream of UAS can be activated by the Gal4 activity.

In 1999, Scheer & Campos-Ortega pioneered the application of the Gal4-UAS technology to zebrafish [51] . Since then, some modified versions of Gal4 have been developed to improve its transcriptional activity, namely Gal4-VP16 and Gal4FF [46], [52]. Gal4FF exhibits lower transcriptional activity than Gal4-VP16 but it is better tolerated in vertebrates because the high levels of Gal4-VP16 expression can suppress transcription of the target gene [46].

The Gal4-UAS transactivation system comprises two components: Gal4 driver and UAS reporter/effector. The generation of fish lines with each component separately increases the possible promoter/reporter combinations by simply crossing the two lines, making this system highly versatile. In detail, the driver lines express Gal4 activator under the control of a tissue-specific promoter whereas the reporter lines include an expression cassette with a Gal4-specific binding sequence (UAS), a minimal promoter, and the gene of interest [8], [45], [51], [53]. When fish carrying Gal4 driver are crossed with others carrying a UAS reporter/effector, gene expression is accomplished in the offspring that contains both alleles (Figure 1.5).

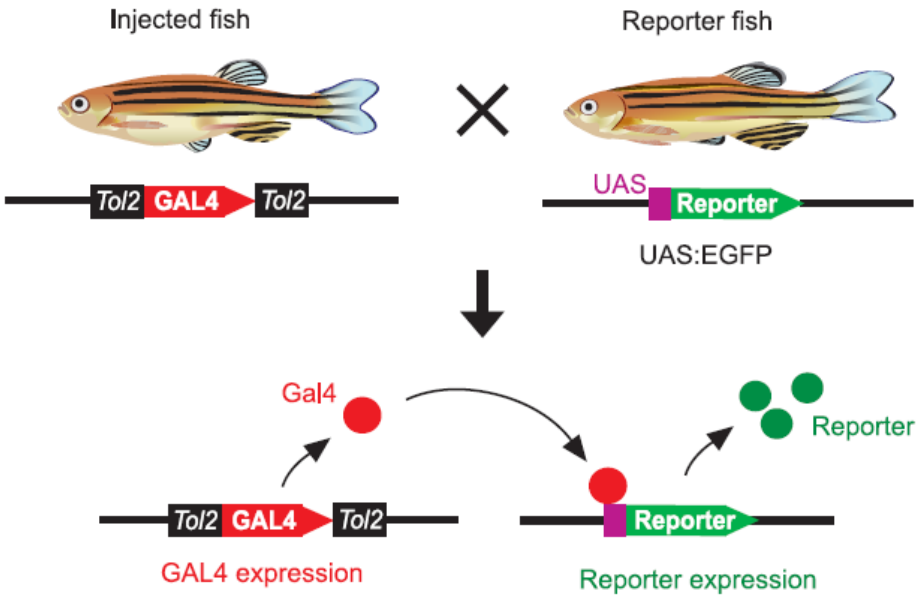

Figure 1.5- Tol2-mediated Gal4-UAS transactivation system in zebrafish. A driver line with a specific promoter upstream of the gal4 gene crossed with a reporter/effector line carrying the gene of interest (EGFP) under the

control of UAS, results in a double transgenic offspring. EGFP is expressed under the control of the driver line's promotor. Adapted from [46].

The gene of interest in the reporter/effector line is expressed according to the expression pattern of the promoter in the Gal4 driver line. For example, promoters as elavl3 (also called HuC) have a pan-neuronal expression pattern and are used to drive expression in all neurons throughout the nervous system [54]. Other promoters, like islet2b (isl2b), are usually used to drive expression to specialized regions, for instance retinal ganglion cells, hindbrain, trigeminal nerve, spinal cord and optic tectum [23].

The Gal4-UAS transactivation system had been extensively employed in genetic research in *Drosophila melanogaster*, however, it was applied in vertebrates only when an efficient transposon system was developed in zebrafish, the Tol2 (section 1.2.1). Taking advantage of the Tol2-mediated Gal4/UAS transactivation, Gal4 gene trap and enhancer trap methods were developed resulting in an expanded collection of transgenic Gal4 and UAS zebrafish lines [46], [53]. This system allows for numerous combinatorial possibilities promoting the generation of countless transgenic zebrafish lines [55]. The applications of such lines include the investigation of anatomy and connectivity of the nervous system and identification of neuronal circuits associated to behavioural-specific responses [45], [51], [53].

1.3 Genetically encoded fluorescent reporters

Genetically encoded fluorescent proteins can be expressed in zebrafish in a cell- or tissue-specific manner or the whole organism using transgenic techniques [19], [53]. The breakthrough and development of various fluorescent reporter variants has transformed studies of/in living systems [56]. Outstandingly, they have the ability to be genetically fused to proteins with specific subcellular tags, limiting fluorescence to specific organelles or structures [55], [57]. These features associated with zebrafish embryos and larvae optical transparency allow *in vivo* studies in real time. Since green fluorescent protein (GFP) discovery, multiple fluorescent reporters have been discovered and developed. Nowadays, a wide collection of proteins spanning the spectrum from ultraviolet to infrared, set the foundation for multicolour imaging [58]. Besides, fluorescent proteins characteristics have been enhanced to achieve higher brightness, faster folding, photo-convertibility, -stability and -activatability and no dissonance excitation/emission properties [55], [56].

1.3.1 Genetically encoded calcium indicators

Calcium ions are universal second messengers engaged in the regulation of a variety of physiological phenomena including muscle contraction, neuronal transmission, fertilization and hormonal secretion [59], [60]. Fluctuations in intracellular calcium concentration are a reliable readout of ongoing neuronal activity as calcium ions are transported into neurons all through action potential firing and synaptic input [23], [61]–[63]. One method for visualizing such fluctuations *in vivo* is through the use of genetically encoded calcium indicators (GECIs), that allow neural activity imaging in a cell-type specific manner [23], [64]. Neuronal circuitry in flies, worms, fish, and humans has been imaged using such indicators.

Single fluorescent protein-based GECI comprise a Calcium-binding domain derived from calmodulin (CaM) or troponin C; a peptide domain that binds the Calcium-binding domain; and a fluorescence protein (FP) domain which fluorescence features are modulated by the Calcium-sensing interaction [59], [65]. Upon calcium binding, the calcium-binding domain (CaM or troponin C) undergoes a conformational change that promotes an intramolecular rearrangement, resulting in higher fluorescence intensity [60], [64], [66]. GCaMPs (Green fluorescence protein (GFP)/Calmodulin (CaM) Protein, GCaMP) are the most extensively used single fluorescent protein-based GECIs. They have, in general, low degradation and high per-molecule brightness [65]. The GFP is fused to the CaM on the C-termini and a muscle myosin light chain kinase M13 peptide on the N-termini (Figure 1.6) [23], [67], [68]. When calcium is present, it binds to CaM, which then undergoes a conformational rearrangement and binds the M13 peptide. This alteration protects GFP from the surrounding aqueous environment, modifying its protonation state in a way that increases its fluorescence [23], [65], [67], [68]. In the presence of calcium ions, GCaMP has a maximum excitation wavelength of 484 nm and maximum emission wavelength of 507 nm [23], [63], [69], [70].

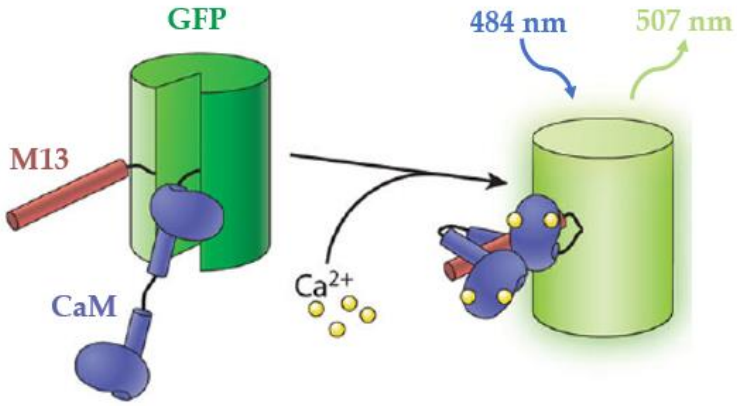

Figure 1.6- Mechanism of the genetically encoded calcium ion indicator GCaMP. Upon calcium binding to the CaM-domain, the CaM-M13 interaction undergoes a conformational rearrangement that reduces GFP contact with the solvent, thus changing the GFP protonation state which leads to the emission of green fluorescence. Adapted from [57].

The GCaMP class of GECI offers yet another advantage, the ability to regulate the colour of the fluorescent protein, usually through mutations directed to the GFP, generating chromophores with distinct spectra properties such as yellow, red and cyan, that can be used for generating alternative GECIs [62].

For efficient detection of neuronal activity, a GCaMP must be highly fluorescent and sensitive both in terms of signal-to-noise ratio and kinetics response. The first version of GCaMP was far from ideal, so many versions have been developed to introduce amino acid changes to produce more sensitive and bright variants of GCaMP [8], [65], [68].

Through a comprehensive mutagenesis study, Michael Orger's Laboratory (at Champalimaud Foundation) has produced new GCaMP variants. One of those, designated GCaMP6fEF05 was produced by combining the 6f variant from the Janelia Farms jGCaMP6f with the GCaMP3 EF05 variant from the Wang lab. In the 6f variant, "f" stands for fast kinetics, a result from focused mutagenesis on CaM-domain and CaM-M13 interface affecting calcium affinity [68]. In the GCaMP3 EF05 means it was the fifth mutant variant with a mutation in one of the EF hand-loops (intrinsically connected to calcium-binding affinity), particularly in III loop [65]. This new variant GCaMP6fEF05 exhibits better signal-to-noise ratio due to high affinity, characteristic of the EF05 variant, while having a fast kinetics, characteristic of the 6f variant. In addition, it seems to exhibit a baseline brighter fluorescence (unpublished work).

1.3.2 LSSmOrange

Multicolour imaging makes use of FPs and is a potent approach to learn the molecular processes in living cells [71]. Despite the availability of distinct FP variants, there is still a need

to develop fluorescent probes with new spectral properties [71]. Approaches exploiting single-excitation wavelength for a class of fluorescent probes with resolvable emission spectra are particularly attractive. Filling up the existing spectral gap between the green-yellow and red Light-induced Spectral Shift Fluorescent Proteins (LSSFPs), a bright fluorescent protein was developed and named LSSmOrange [71].

LSSmOrange is a monomeric orange fluorescent protein (mOrange) exhibiting a large spectral shift between the excitation and emission peaks, designed Light-induced Spectral Shift (LSS) [58], [71], [72]. LSSmOrange presents excitation and emission maximum wavelengths of 437 nm and 572 nm, respectively (Figure 1.7). It is brighter than the brightest red LSS fluorescent protein, and similar to green-yellow LSS fluorescent proteins brightness [71]. It has been reported the possibility of LSSmOrange undergoing photoconversion, resulting in alterations in the maximum excitation wavelength of the chromophore (from 437 to 553 nm) with no changes in the emission spectrum [58], [71], [72].

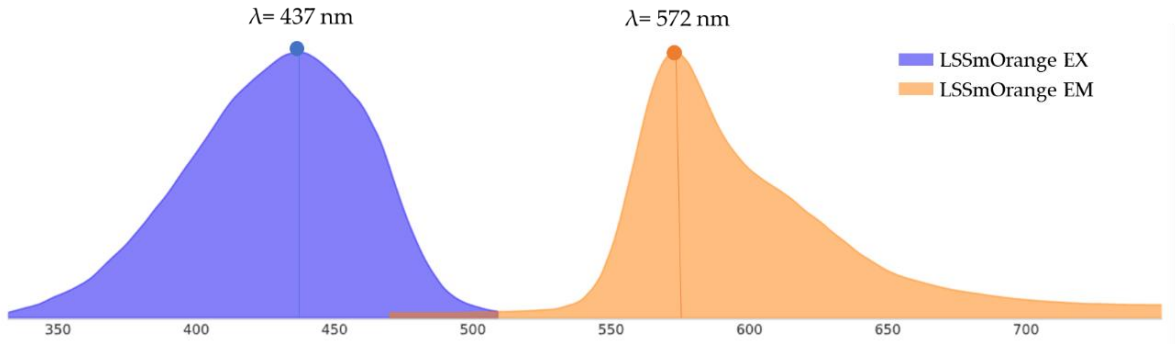

Figure 1.7- LSSmOrange fluorescence spectrum. Represented in blue, the excitation spectra with the maximum wavelength at 437 nm. In Orange, the emission spectra with the maximum wavelength at 572 nm. Adapted from FPbase (https://www.fpbase.org/).

The well-separated excitation and emission spectra (stoke shift) of LSSmOrange, typically above 100 nm, offers a weighty advantage in multicolour fluorescence microscopy with single-laser wavelength [72], [73]. Thanks to differences in the stokes shifts, there are FPs emitting at well-separated wavelengths subsequent to excitation with a single wavelength, when co-expressed. Multicolour applications for these long shifted fluorescent proteins include single-laser dual FRET and flow cytometry [56], [72], [73]. In addition, the use of a single-excitation wavelength offers the opportunity as well to study various processes in real time [57], [71], [73]. The advantages of LSSmOrange expanded prospects of fast multicolour imaging and became a probe of preference to follow and measure several populations of intracellular components [71].

1.4 Molecule's delivery methods into organism/cell of interest

Generally, there are three types of gene delivery methods for effective delivery of foreign DNA to target cells and organisms *in vivo*: (1) biological, (2) chemical and (3) physical approaches. Biological approaches use viral vectors to transfer genes, but this present safety issues that impose tight quality control standards. Additionally, the price and storage conditions are limitations to using biological vectors in ordinary clinical practice [74], [75]. The chemical methods consist of delivering the genetic material through the use of agents such as cationic lipids and polymers, which presents an advantage when it comes to safety, large-scale production and costs. However, the cell type, reagent formulation, and DNA/reagent ratio, among other factors, all have repercussions on transfection efficiency [74], [75]. The preponderance of physical approaches comprises the use of an instrument, resulting in more controlled and standardized gene delivery. The delivery of DNA through a variety of physical methods, such as electroporation and microinjection, require the application of external forces (electric pulses and pressure, respectively) for effective molecules delivery into target cells or tissues. Physical techniques, in contrast to biological and chemical techniques, allow transfection in all cell types with greater efficiency, have fewer gene size constraints and are easily standardized [74], [75]. Microinjection and electroporation are currently the two most prevalent techniques for delivering foreign molecules such as DNA, RNA and proteins into cells or tissues.

1.4.1 Microinjection

Microinjection is a direct method to introduce foreign materials into specific targeted cells and tissues [75], and has been carried out since the beginning of the 20th century [76]. By physically piercing the cell membrane with an injection needle and injecting substances into targeted areas, single cell microinjection can deliver proteins, peptides, cDNAs, or large molecule non-diffusible drugs effectively into cells. The standard microinjection settings include an inverted microscope, a light source, injection needles and microinjectors to provide pressure to inject materials into cells or tissues. In addition a micromanipulator can be used to control the needle movement and position the needle where the injection is going to take place [77]. The manual microinjection offers greater flexibility of movement and so it is faster; however, it depends on the operator skills for the cells and tissues not be damaged [75]. The micromanipulator is a device used to maximize needle stability, increasing the cells and tissues viability [74]–[76].

Expulsion of solution containing the genetic material through the glass capillary needle uses hydrostatic pressure [74], [76]. The small tip diameters of these needles allow accurate and precise delivery [74]. Upon material successful delivery, subsequent biochemical reactions are expected to occur and produce the desired result, such as gene-specific editing, random-integration of transgenes into the host genome, blockage of mRNA translation, tissues staining, etc. [76].

Microinjection has a wide range of applications, it can be used to produce transgenics and gene editing when applied to zygotes but it can also be used in adult organisms, for example to deliver drugs, dyes or morpholinos in specific tissues [76], [77]. It is a technique for generating transgenics reported in several organisms such as *C.elegans* [78], mouse [79], [80], porcine [81], xenopus [82] and zebrafish [83]. In addition, is a technique used in certain clinical applications such as the treatment of infertility with intraplasmatic sperm injections and the treatment of genetic diseases caused by mutations in mitochondrial DNA [74]. In zebrafish research, microinjection is a crucial technique that has been utilized to create a diverse range of zebrafish mutant and transgenic lines. Microinjection of embryos at particular early stages (1 to 4 cell stages) was used to establish the bulk of these lines. However, injection into later stage zebrafish larvae has been successfully used for numerous functional research in recent years. Furthermore, immune-compromised adult zebrafish were given hematopoietic, muscle stem cell, and cancer cells to see whether conditions were best for long-term cell engraftment [83]. Microinjection is used to change the genomic DNA sequence irreversibly altering the function of the targeted gene and accomplish random genomic mutagenesis by retroviral DNA integrations or transposable elements with gene- or enhancer-traps. In addition, is used to cause loss-of-function by injection for examples of morpholino (MO) and gain-of-function injecting synthetic mRNAs that will be translated to protein. Moreover, has been used to explore gene function by changing the levels of protein translation temporarily [74].

Advantages of the method include the simplicity of the approach in terms of conception, the possibility to precisely control the volume of test substances delivered, and high delivery success rate [75], [76], [84]. Each time a new needle is used, it requires calibration, using a pointed iron forceps and keep adjusting the air ejection pressure to obtain the desired volume of solution injected with each pulse of air. Volume control is however not required for all applications. However, it is a time-consuming procedure, especially when injecting cells, because only one cell can be injected at a time [76]. Invariably, just a limited number of cells can be transfected per experiment, and achieving a successful transgene expression requires some injection repetitions [74]. Furthermore, the physical damage to the cell's membrane,

during the microinjection impacts cells survival rates [85]. Moreover, the manual microinjection relies not only on needle's quality but also on the operator skills and sensitivity, which makes the technique less reproductible [86]. Cell death can be kept to a minimum if high-quality micropipettes and good technique are used; however if needles are too large or irregular cell death can occur after the needle is removed [76]. To overcome some of microinjection limitations, more advanced technology has been developed over time to improve micromanipulators and injectors by incorporating automation, which leads to a greater survival rate and also makes the technique more reproducible [86], [87].

1.4.2 Electroporation

Electroporation is a biophysical transfection method where permeabilizing structures, nanopores, are assembled in the cell membrane in response to an imposed pulsed electrical field to amplify cell membrane permeability to the transportation of ions, molecules and foreign substances [74], [77], [88]–[95].

The electroporation method is divided into two categories: reversible electroporation and irreversible electroporation. Reversible electroporation is characterized by the formation of transient nanopores that only last between seconds and minutes after which the cell membrane revenues to its basal condition [74], [89]–[93]. This phenomenon includes reversible cell electro-permeabilization followed by mass transfer of non-permeant molecules of interest across the cell membrane. The transient permeabilization is advantageous for several applications, including gene, therapeutic agents and drug delivery used for example in the generation of transgenic lines, molecular therapy and electrochemotherapy, respectively [88]. In opposition, irreversible electroporation is characterized by the permanent permeabilization of the cell membrane due to the appliance of intense and/or long electrical pulses [74], [89]–[93], thus preventing nanopores to reseal, leading to an osmotic imbalance and ultimately cell viability compromise. By ensuring cell death, irreversible electroporation has become a promising biomedical technology, and its applications include bacterial inactivation and tumour ablation. The difference between the two types of electroporation is the magnitude of the applied electrical pulse, meaning the voltage used along with the number and duration of delivered pulses [91].

To better understand the electroporation principle, one should understand basic electrical characteristics of the cell membrane. The cell membrane resembles an electrical circuit with capacitance by storing charge [93]. It is composed by phospholipidic bilayers; each phospholipid comprises a hydrophilic lipid head and a carbonated hydrophobic tail. The

phospholipids are displayed with hydrophilic heads turned to the inner and outer side of membrane whereas the hydrophobic tails are located in between [74], [88]–[90], [93], [96]–[98]. In the presence of a pulsed electrical field, the dipole water molecules surrounding the cell membrane undergo a reorientation and form a thick water column that penetrates the phospholipidic bilayer (Figure 1.8) [97], [98]. This infiltration causes the phospholipids disposition rearrangement, in order to prevent the hydrophobic carbonated tails to contact with the solvent [98]. In turn, it causes the water column to enlarge which leads to hydrophilic larger pores to form resulting in a permeation event that allows water and other charged molecules to enter the cell. Upon electric field removal, the permeable structures, pores, destabilize leading to its closure usually within seconds to minutes, allowing the cell membrane to reseal [97].

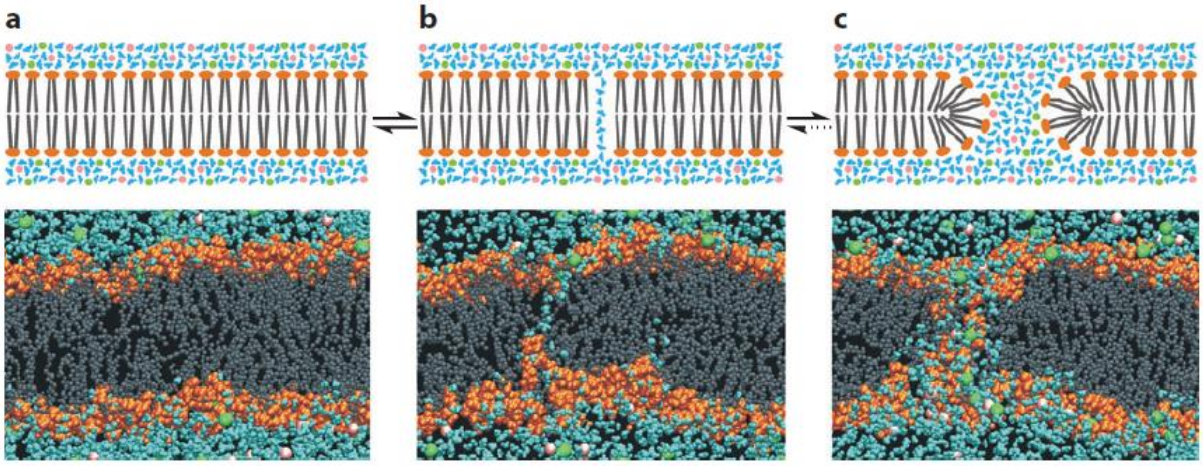

Figure 1.8- Cell membrane rearrangement upon a pulsed electrical field (EF) appliance. Top panels: molecular scheme. Bottom panels: atomic simulation of molecular dynamics. (a) Bilayer membrane in resting state; (b) Dielectric water molecules penetrating the cell membrane in the presence of an EF; (c) rearrangement of phospholipids close to the water molecules penetrating the membrane in an attempt to preserve the homeostasis, resulting in enlargement of water column forming the pores, allowing charged molecules to enter the cell. Colour code: atoms from the hydrophilic heads (orange); atoms from the hydrophobic tail of phospholipids (grey); water molecules (cyan); sodium ions (green) and chloride ions (pink). Retrieved from [95].

The electroporator and electrodes are required and crucial equipment for electroporation. Depending on the application, electrodes can be incorporated into an electroporation chamber, such as in the case of electroporation of mouse embryos or cell cultures, in which the cells or embryos are placed in the chamber with a conductive medium that allows transfection without direct contact with the electrodes. In opposition, pinch-type electrodes can also be used, for example in chicken embryos or muscle tissue electroporation, where the electroporation paddles are placed directly over the desired location on either side of the tissue. In this last case, microinjection is employed first, followed by electroporation.

Due to conditions such as temperature in the laboratory, which regulates membrane permeabilization by acting on the fluidity of membrane lipids, the same protocol applied at different sites can result in completely different efficiencies [96]. As a result, protocol optimizations are always required as a first approach or whenever an experimental condition is altered. The electroporation transfection efficiency and cell viability rely on numerous factors from electroporation conditions to parameters. A wide number of protocols have been created for a variety of applications. The purpose of the experiment, the type and area of cell/tissue to be electroporated, the cell stage in the case of zygote electroporation, the type of molecules to be transferred, and other factors must all be considered while choosing an electroporation protocol [88], [99]. Only by properly optimizing the electroporation parameters may considerable improvements in experimental outcomes be achieved [97], [98]. A few essential parameters include, electrical field, number and duration of electrical pulses, buffer solution and DNA concentration [74][99]. Electric field distributions are important for achieving high cell viability since they can lead to irreversible pore formation and cell death. The pore density is influenced by the field intensity, while the pore size is influenced by the pulse duration [100]. Because it works as a current conductor, choosing the right buffer solution is crucial. High ionic strength buffers, such as PBS (Phosphate-buffered saline), are preferred [96]. The pH of the buffer should always be close to that of the cell environment, which is around 7. Moreover, electroporation can occur in a single pulse system or a multiple pulse system. Furthermore, controlling the system's impedance/resistance values is critical since it affects the field perceived by cells and tissues and can result in electroporation inefficiency [96].

Electroporation is a fast, cost effective, easy to employ and versatile transfection technique [88], [91], [101]. It is the chosen method for the transfection of difficult-to-transfect cargos (due to their size) and cells (owing to their complexity) [99]. Furthermore, it can be used to transfect a large number of cells simultaneously in a short period of time [74], [89], [90].

In the past 30 years, this delivery method has progressed beyond isolated-cultured cell transfection such as fungi, bacteria and even human cells in culture to living tissue transfection in animals, such as chick, xenopus, mice and even in humans [74], [92], [101], [102], [89], [92], [95], [103]. Nowadays, electroporation is extensively used in biomedical and biotechnological research, with new clinical and industrial applications for both reversible and irreversible electroporation constantly emerging [92], [98]. Applications range from electrochemotherapy, gene therapy, DNA vaccines, clinical diagnosis, therapeutics and tumour ablation, to cell fate mapping, neuronal activity monitorization, generation of transgenic and mutant lines,

depending on the organisms, tissues or cells to which it is applied [77], [88], [91], [98], [102], [104], [105].

In zebrafish, electroporation has been employed mostly to deliver charged molecules to specific tissues in larval and adult fish. It has been used to insert DNA constructs, dyes, etc, into neurons, muscle and retina, to express a broad diversity of transgenes including GECI, fluorescent proteins and optogenetic probes [101]. However, there is a developmental stage gap in the use of electroporation in zebrafish, since only a few laboratories explored this technique in embryos. In addition, most publications are outdated in relation to the devices used, that have now been improved, as well as in relation to scientific knowledge.

1.5 Aims

The main aim of this work was to optimize the *in vivo* electroporation technique for delivery of exogenous genetic material into zebrafish embryos and compare its efficiency to the most widely used delivery method, microinjection. In parallel, work on the generation of a new neuronal zebrafish line, with an enhanced genetically encoded calcium indicator reporter, namely, GCaMP6f EF05. The specific aims of this project are:

1. Optimization of *in vivo* electroporation and comparison to microinjection as delivery methods for transgenesis in zebrafish:
    a. Optimization of the dechorionation method
    b. Selection of the cell stage in which embryos are electroporated
    c. Optimization of the electrical pulses (Poring and Transfer)
    d. Optimization of the DNA solution medium and DNA concentration delivered
    e. Comparison of the efficiency of the optimized electroporation protocol with the previously established microinjection
2. Generation of a new neuronal zebrafish line:
    a. Generation of a stable transgenic zebrafish line by injecting the elavl3:GCaMP6f EF05 construct into one-cell stage zebrafish embryos, using the Tol2 transposon system
    b. Establishment of the line throughout generations
    c. Confirmation of the pan-neuronal expression pattern by confocal imaging

2

MATERIALS AND METHODS

2.1 Zebrafish husbandry

Zebrafish husbandry at Champalimaud Fish platform was performed according to what is described in Martins *et al.* 2016, with adaptations based on Monteiro *et al.* 2018 [106], [107]. Briefly, the fish holding rooms were kept at a controlled temperature of 25 °C and humidity levels between 50 % and 60 %. The room's automatic photoperiod was set to 14-hour light/ 10-hour dark, with the "day" period starting at 8:00, and light intensity was maintained between 200 and 300 lux at the water surface. Fish were maintained in Tecniplast® Centralized Life Support (CLS) recirculating systems at a maximum density of 10 fish per litre. Water conductivity was kept at ~1300 µS, temperature at ~28 ºC, pH ~7.4 and total dissolved gases <0 and the four parameters were controlled daily. Other water parameters were checked every week including ammonia, nitrites, nitrates. Embryos and larvae up to 5 or 6 dpf were housed in dedicated incubators at 28ºC and photoperiod of 14-hour light/10-hour dark. Fish were feed 2, 3 or 4 times per day depending on the developmental stage, with live feeds (rotifers or artemia sp.) and formulated dry pelleted feeds Zebrafeed®Sparos and Gemma®Skretting, as detailed described elsewhere [106], [107].

When organisms where no longer of use for additional experiments, they were euthanized. Euthanasia was performed as described in Martins *et al.*, 2016 [106].

2.2 Zebrafish strains

In this project experiments, three zebrafish strains were used: Tübingen (TU), nacre$^{-/-}$ and Tg(isl2b:Gal4$^{+/+}$). TU is a well-known wild-type line. Nacre$^{-/-}$ are recessive mutants with a complete absence of melanophores. This is due to a single-base mutation in the microphthalmia-associated transcription factor a (*mitfa*) crucial for the melanophores' development. Tg(isl2b:Gal4$^{+/+}$) is a driver line that triggers Gal4 expression under the control

of the Isl2b promoter and contains a heart specific GFP marker, very useful for identifying fish that carry this construct [108].

2.3 Zebrafish crosses, embryo collection and care

All crosses were set using adult fish older than 3 months. Crosses were set between 15:00 and 18:00, in breeding tanks composed of an external tank, internal tank with perforated bottom and lid. The internal perforated bottom allowed eggs to pass through and accumulate on the bottom of the outer tank, where progenitors could not access, thus preventing the eggs from being eaten by the adults [109]. The next morning, after spawning, embryos were collected with a strainer, transferred to petri dishes containing embryonic medium (E3) and used for experiments. After that, embryos were kept at a density of ~50 embryos per plate, in dedicated incubators. At ~24 hours post-fertilization (hpf) the non-viable embryos were removed and the E3 in the plates replaced. The spawners were housed back into their holding tanks upon embryo collection, and crossing frequency was one time per week, to avoid compromising fish welfare and offspring quality and quantity.

When embryos were to be raised, they were subjected to a surface disinfection protocol (bleaching) at 1 dpf following an established protocol [106], to reduce the risk of pathogen transmission within the fish population. Larvae were transferred to the nursery in a recirculating holding system, at 5 or 6 dpf, to be raised to adulthood.

2.3.1 Crosses for microinjection and electroporation

Tg(isl2b:Gal4$^{+/+}$) fish were crossed for obtaining embryos for microinjection and electroporation of 10xUAS:LLSmOrange. TU and Nacre fish were crossed to generate embryos used for microinjection of elavl3:GCaMP6f EF05 DNA construct.

Crosses were set in 2 L breeding tanks, using a female-to-male ratio of 5:3. A vertical tank divider was inserted on each tank to keep males and females apart and was removed the following morning to allow for spawning in a controlled manner. Ten minutes after spawning, embryos were collected and immediately used for injections or electroporation.

2.3.2 Crosses for evaluation of germline transmission of delivered constructs and selection of founders (F0) and F1

Each adult fish to be screened was crossed, in a 1 L breeding tank, with one TU or nacre$^{-/-}$ fish (whichever corresponded to the opposite pigmentation of the fish of interest) of opposite gender. The following morning, the crossing pairs that did not breed were returned to the original holding tanks. For pairs that produced offspring, the TU or nacre$^{-/-}$ were returned to the original tanks whereas the fish of interest were housed individually and given a unique identifier code. Embryos from each cross were collected separately, the plates identified with the same identifier as the parent of interest. Embryos were screened at 3-5 dpf, and depending on the result, the correspondent parent of interest was maintained or euthanized.

2.3.3 *In vitro* fertilization (IVF)

The IVF technique was employed to obtain embryos from seven fish from founder populations of the elavl3:GCaMP6f EF05 transgenic line (four males and three females) that had not produced offspring through natural mating for three consecutive weeks.

2.3.3.1 Crosses

To stimulate gametes production, each founder fish was crossed and housed as described in section 2.3.2. In parallel, two bulk crosses of 5 female:3 male ratio of wild-type fish were also set, as described in section 2.3.1. These wild-type crosses aimed at stimulating gamete production, that were used to fecund founder's gametes.

2.3.3.2 Female hormonal stimulation

In the morning after crosses set-up, females were individually transferred to breeding boxes filled with 15 µL of 100 nM β-Dihydroprogesterone hormone (β-DHP; Solution's chapter) per 100 mL of system water in a total of 500 mL, where were left to incubate for 4 to 5 hours.

2.3.3.3 Sperm collection

While the female hormonal stimulation was in place, to harvest the sperm, 10 µl of Aquaboost® Spermcoat were added to 0.5 mL Eppendorf tubes and placed in a styrofoam ice box. Then, each male was anesthetized, rinsed in Phosphate Buffered Saline, pH 7.2 (PBS; Solution's chapter), the urogenital pore area dried with a Kimwipe and finally sperm collected, all as described in Draper & Moens, 2009 [110]. The overall set up is represented in Figure 2.1. 2-3 µL of sperm of each male was collected and immediately expelled into the Eppendorf tube

with the chilled Aquaboost® Spermcoat. The male was placed in a recovery tank with fresh fish water before returning to the holding tank. In parallel, the sperm quality was checked based on the colour, density and motility, and samples were kept on ice.

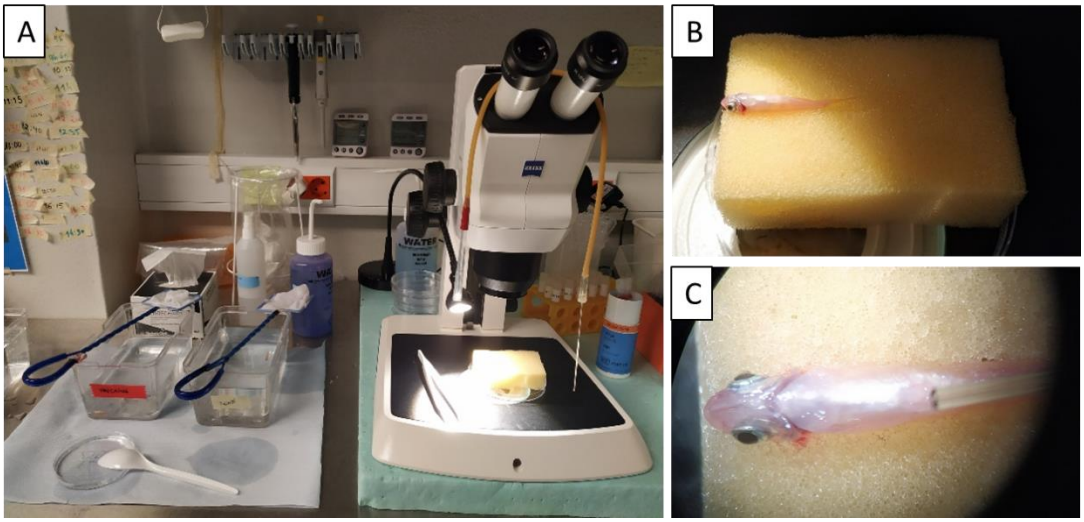

Figure 2.1- Set-up for sperm collection. (A) Overview of the sperm collecting set-up. Starting on the left, an anaesthetic bath containing 500 mL of 0.6 mM Tricaine; next is a recovery bath with fresh system water, and on the front, a petri dish containing 1x PBS for rinsing the fish. On the right, there is a sponge where the males were placed for sperm collection into a capillary through suction while using forceps pression and stroke on the male sides. All this under a stereoscope (Zeiss SteREO Discovery.V8) (B) Detail of a male positioning right before sperm collection. (C) Capillary placement to spread the pelvic fins apart, access the urogenital pore and extract the sperm.

2.3.3.4 Oocyte collection

After hormone incubation, females were anaesthetised, rinsed in PBS, the cloacal area was gently dried and finally oocytes were collected as described [111] and depicted in Figure 2.2. Oocyte's quality was quickly checked - clutches that looked whitish and with turbidity, dirt or watery eggs were not used. Good quality eggs were immediately transferred to a petri dish for *in vitro* fertilization (see next section). The females were placed in a recovery tank filled with fresh fish water until full recovery from the anaesthesia, and then housed back to the original tanks.

2.3.3.5 Fertilization of Oocytes

Soon after collecting the oocytes into a dry petri dish, 10 µL of collected sperm in Aquaboost® Spermcoat and 200 µL of fish holding water were added and left to incubate for 2 to 5 minutes at room temperature, for gametes activation and oocytes fertilization. Then, the petri plate was filled with fish water and housed in the dedicated incubator. The fertilization rate was assessed the next morning, along with plate cleaning and water replacement.

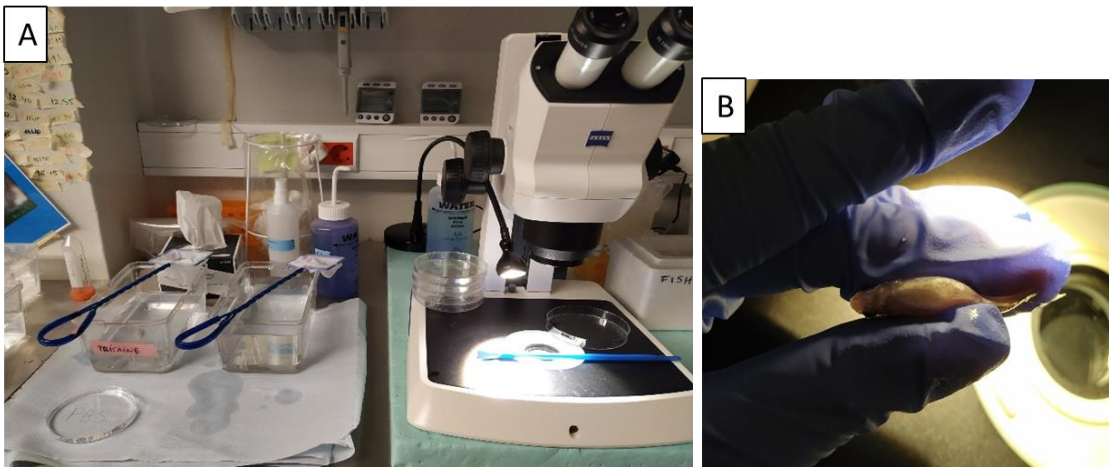

Figure 2.2- Setup for egg harvest. (A) Complete female gamete collecting setup. An anaesthetic bath containing 0.6 mM Tricaine in 500 mL of system water is depicted on the left, followed by a recovery bath with fresh system water, and on the front a petri dish with 1x PBS for female rinsing after anesthesia. On the right is a polypropylene spatula for oocytes collection, the petri dish where they were transferred to, and the stereoscope (Zeiss SteREO Discovery.V8) used to inspect the egg clutch's quality. (B) Detail of positioning of a female for gametes collection by gently pressing the ovaries.

2.4 Microinjection of zebrafish embryos

Microinjection is the most widely used delivery method for generating transgenic zebrafish and relies on the injection of the exogenous molecules of interest directly into one-cell stage embryos (0 to 45 minutes post fertilization).

2.4.1 Needles

Microinjection needles (Figure 2.3C) were made from borosilicate glass capillaries (World Precision Instruments Inc., TW100-4), using a Laser-Based Micropipette Puller (Sutter Instrument CO., Model P-2000) with the following settings: heat = 400, filament = 4, velocity = 45, delay = 200 and pull = 100.

2.4.2 Microinjection plates

1% agarose (w/v) was prepared by adding 1g agarose to 100 mL E3 and heating this solution in a microwave oven until all agarose was dissolved. The solution was distributed into two petri plates with 10 cm diameter, and a mould was placed on each plate, the grooved mould surface facing down. When the agarose solidified, the moulds were removed, exhibiting the agarose grooves used to align and hold embryos in place during injection (Figure 2.3 B and D).

2.4.3 Microinjection mixes

For generating a stable zebrafish transgenic line for elavl3:GCaMP6f EF05, the following injection mix was prepared for each injection session (Table 2.1).

Table 2.1- Concentrations and volumes of the different components of the elavl3:GCaMP6f EF05 injection mix

Stock solution	Volume of stock solution	Concentration in injection mix
500 ng/µL Tol2 mRNA	2 µL	100 ng/µL
100 ng/µL recombinant plasmid DNA with elavl3:GCaMP6f EF05 insert	5 µL	50 ng/µL
phenol red (pH-sensitive dye)	5 µL	n/a
Final volume	10 µL	n/a

For testing the 10xUAS:LSSmOrange plasmid quality and efficiency before using it for electroporation optimization, it was first injected using the mix described in Table 2.2.

All injection mixes were kept on ice until fish spawned and there were 1-cell stage embryos available to inject.

Table 2.2- Concentrations and volumes of the different components comprising the injection mix used to generate the 10xUAS:LSSmOrange transient transgenics

Stock solution	Volume of stock solution	Concentration in injection mix
100 ng/µL recombinant plasmid DNA with 10xUAS: LSSmOrange insert	1.8 µL	18 ng/µL
phenol red (pH-sensitive dye)	8.2 µL	n/a
Final volume	10 µL	n/a

2.4.4 Set-up and injections

The microinjection needle was loaded with 2 µL of injection mixture using 20 µL Eppendorf Microloader™ tips (FISHER) and carefully inserted into the PicoNozzle tip (Figure 2.3A). Next, the zebrafish embryos were aligned in the grooves of the injection plate (Figure

2.3D) using a 3 mL Pasteur pipette with some drops of E3 medium to prevent the embryos from drying. Finally, the tip of the injection needle was broken, using forceps (FST by DUMONT Biology), and the injection was performed under a stereoscope (Zeiss SteREO Discovery.V8) with a PV 820 Pneumatic PicoPump (World Precision Instruments Inc.) (Figure 2.3B) under the following settings: pressure on, vent on, gated on, a range of 100 ms, hold pressure of ~0.25 psi and eject pressure ~10 psi. 10xUAS:LSSmOrange injection mix was delivered to Tg(isl2b:Gal4+/+) embryos and elavl3:GCaMP6f EF05 mix was injected in nacre+/- embryos. The needle softly perforated the chorion until reaching the cell where the injection mix was then delivered through a short burst of air. Once all embryos were injected, they were gently transferred to a petri dish with fresh E3 medium and incubated overnight. Non-manipulated embryos were also kept in the incubator for control purposes. At 24 hpf the total number of embryos was counted, as well as non-viable/dead ones, and used to calculate survival rate.

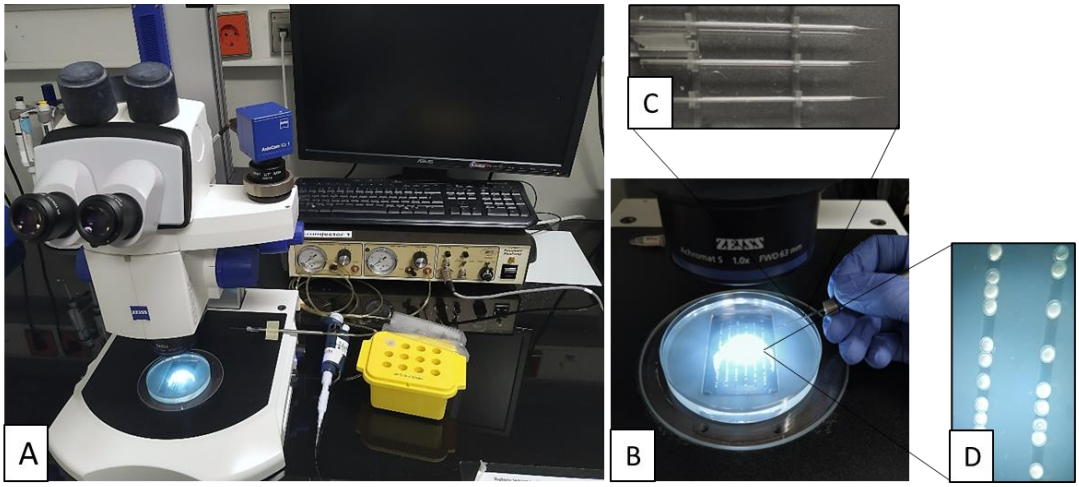

Figure 2.3- Set-up for the microinjection into one-cell stage zebrafish embryos. (A) From letf to right: stereoscope (Zeiss SteREO Discovery.V8) with embryos on 1% agarose plate; microinjection needle attached to the PV 820 Pneumatic PicoPump (World Precision Instruments Inc.); 20 µL micropipette with a 20 µL Eppendorf Microloader™ tip filled with injection mix, to load the injection needle; 1.5 mL Eppendorf with Injection mix in cold-preserving yellow box. (B) One-cell stage zebrafish embryos injection under the the stereoscope. (C) Borosilicate glass microinjection needles. (D) Detail of alignment of one-cell stage zebrafish embryos in the agarose plate trenches.

2.5 Electroporation

Electroporation relies on the application of an external electrical field to enable the delivery of electrically charged exogenous molecules of interest into the host, but the technique is not fully optimed for zebrafish embryos. Methods for pursuing such optimizations are described next.

2.5.1 Equipment

A NEPA21 electroporator (NEPA GENE, Japan) with custom-made blade-type platinum plate Electrodes (CUYX0056, NEPA GENE, Japan) was used for electroporation (Figure 2.4A and 2.4C). This chamber is an adaptation of the CUY520P5 chamber to accommodate a larger number of embryos. It is 15 mm long, 3 mm deep and electrodes are the distance between the two electrodes is 5 mm, which is the width of the chamber. The whole setup is mounted on a glass slide (Figure 2.4C).

2.5.2 Set-up and general protocol

For each electroporation round, a clutch of embryos was dechorionated (Figure 2.4B) and placed inside the electroporation chamber already containing the DNA solution using a cut pipette tip and carefully positioned to prevent contact with the electrodes. A set of experimental electrical pulses were applied (section 2.5.3.5). Poring and transfer pulses voltage varied (section 2.5.3). Constant electroporation parameters were poring pulses of 5 ms pulse length, 50 ms pulse interval, 4 pulses, 10 % voltage decay, and + polarity orientation, followed by transfer pulses of 50 ms pulse, 50 ms pulse interval, 5 pulses, 40 % voltage decay, and ± polarity orientation [112].

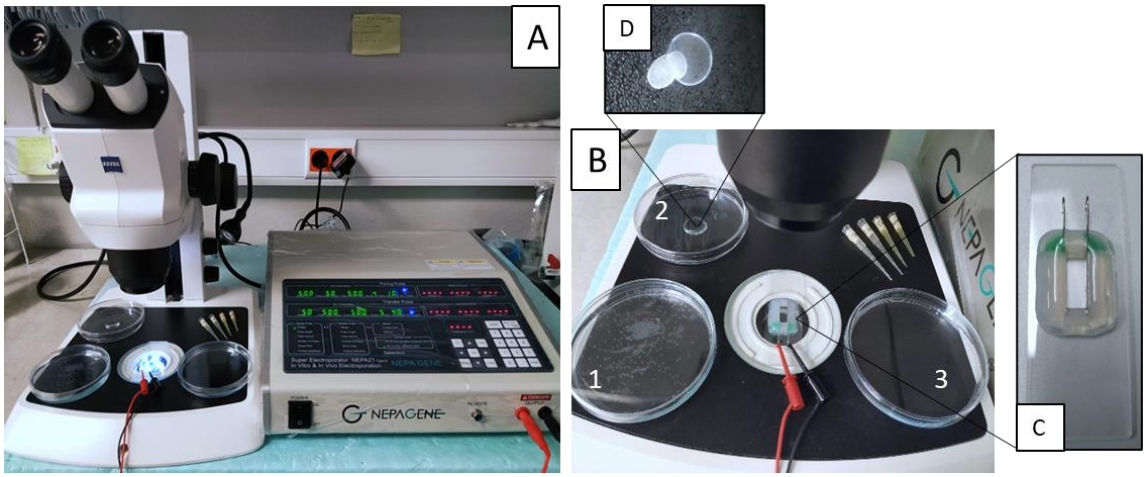

Figure 2.4- Electroporation of zebrafish embryos set-up. (A) A zoomed-out picture of the whole electroporation setup, emphasizing the NEPA21 super electroporator (NEPAGENE) that was used to electroporate the embryos and the stereoscope (Zeiss SteREO Discovery.V8) that was used to handle the embryos. (B) Petri dish in which embryos were taken from (1) and treated for 15 minutes at room temperature in a 500 µL drop of 0.6 mg/L Pronase (2). The embryos were inserted in the electroporation chamber loaded with plasmid DNA using a cut 200 µL pipette-tip after being rinsed three times with 1xEmbryonic medium (E3) and the poring and transfer pulses were administered. Finally, the embryos were cultured overnight in fresh 1xE3 (3) at 28 °C in a 14-hour light/10-hour dark cycle. (C)

Zoomed-in view for a better look at the electroporation chamber (CUYX0056, NEPAGENE). (D) Illustration of dechorionation removal with 0.6 mg/mL pronase incubation at room temperature for 15 minutes.

Embryos were gently transferred, using another cut pipette-tip, into a petri dish filled with fresh E3 medium and kept in a dedicated embryos' incubator. At 24 hpf, the E3 was changed and dead embryos were quantified and removed. The efficiency of electroporation was assessed by calculating survival rates and by screening for positive, fluorescent larvae at 3 to 4 dpf, followed by embryo's transfer to a new petri dish filled with E3.

2.5.3 Optimization of electroporation as a delivery method for transgenesis in zebrafish

2.5.3.1 Dechorionation method

2.5.3.1.1 Mechanic dechorionation

8-cell stage embryos in petri dishes with E3 were placed under light and magnification of a stereoscope and chorions were carefully removed manually using two sharp steel forceps.

2.5.3.1.2 Enzymatic dechorionation

8-cell stage embryos were incubated for 15 minutes at room temperature (~25ºC) with 500 μL of 0.6 mg/mL pronase, so that this enzyme would digest the chorion. Half the embryos were kept under agitation and half without (Figure 2.4B1 and Figure 2.4D). Embryos were washed in E3 and gently rinsed three more times for full chorion removal and pronase elimination.

2.5.3.2 Number of embryos in the electroporation chamber

The optimal number of embryos in the electroporation chamber was determined through try and error taking into account the size of each embryo, the dimensions of the electroporation chamber and the fact that embryos cannot be in direct contact with the electrodes on the sides of the chamber. The amount of 8-cell stage embryos tested in the electroporation chamber filled with E3 medium were 15, 20, 30 and 40 embryos.

2.5.3.3 Volume of the DNA solution in the electroporation chamber

For defining the minimal volume of DNA solution required in the CUYX0056 electroporation chamber, the electroporation chamber was filled with 100 uL (as used by Zhang, C. *et al.*, 2020) of E3, then 30 embryos were placed in the chamber, and more volume was added 10 μL at a time until it was verified that the whole perimeter and embryo's surface

was covered [112]. Once reached that point the minimal volume required of DNA solution was found.

2.5.3.4 Developmental stage used for electroporation

Dechorionated 8- and 1-cell stage embryos were electroporated over the course of 7 electroporation rounds, each comprising 30 embryos, with 10xUAS:LLSmOrange plasmid DNA at 180 ng/μL with the following conditions: poring pulses of 50 V voltage.

2.5.3.5 Electrical pulses

Dechorionated 1-cell stage embryos were electroporated during the course of 7 electroporation rounds each comprising 30 embryos, with 10xUAS:LLSmOrange plasmid DNA at 180 ng/μL. The transfer pulse was set at 10 V and then at 5V, while the poring pulse was set to 50, 75 or 100 V. Survival and efficiency of tested conditions was compared. After optimizing all parameters described on the present section 2.5.3, a new test of poring pulse voltages was performed: the transfer pulse was set at 5 V while the poring pulse was varied between 30, 35 and 40 V, using 10xUAS:LSSmOrange at 300 ng/μL. For all tests, all parameters aside from the pulse's voltage remained unchanged.

2.5.3.6 DNA dilution medium

In order to determine which of the DNA dilution media available in the lab would have less impact on impedance values, which is the resistance of this system, three different dilution media were tested: RNAse free water, MilliQ water and Phosphate Buffered Saline (PBS). The DNA solution was prepared using each one of the previous media and tested, with the concentration set on 180 ng/μL. All solutions were placed in the chamber along with 30 embryos, to simulate real electroporation conditions, and the impedance values were read.

2.5.3.7 DNA concentration

Dechorionated 1-cell stage embryos were electroporated during the course of 14 electroporation rounds each comprising 30 embryos, with plasmid DNA concentrations of 180, 250, 300, 350 and 400 ng/μL. The poring pulses were 50 V voltage, followed by transfer pulses of 5 V voltage.

2.6 Fluorescence screens

2.6.1 Screens for establishment of Tg(elavl3:GCaMP6f EF05) zebrafish lines

To evaluate the success of Tg(elavl3:GCaMP6f EF05) microinjections, 3 to 4 days old larvae were screened for green fluorescence, characteristic of GCaMPs. The positive larvae with the higher fluorescence intensity and complete expression pattern were chosen to be raised until adulthood and represented the "F-1" generation. Once reached sexual maturity, these fish were crossed as described in section 2.3.2 to evaluate in the progeny whether there had been germline transmission of the construct to the offspring. The F-1 fish whose progeny included larvae with a strong (in intensity) and complete expression pattern for Tg(elavl3:GCaMP6f EF05) (pan-neuronal, expression in all nerve cells) were chosen as breeders to grow founder (F0) populations. When F0 fish were adults, they were crossed and similar screens were done to the progeny, to select among the F0 population, the best individual Tg(elavl3:GCaMP6f EF05) founder fish, whose progeny (F1) was used to establish stable transgenic fish lines.

All screens were repeated at least twice, after two cross rounds, and additional crosses were done until at least 200 larvae from each adult fish under screen were obtained.

2.6.2 Screens for 10xUAS:LSSmOrange plasmid validation and for evaluating electroporation efficiency

Injected or electroporated larvae were screened between 3-4 dpf for transient expression (in the form of orange fluorescence) in the trigeminal nerve, retinal ganglion cells, hindbrain, spinal cord, and optic tectum, which is the characteristic expression pattern of the isl2b promoter line used for the UAS injections and electroporation. Attention was given to the level of expression (complete or incomplete) and to the intensity of the fluorescence. Total number of larvae and number of larvae positive for the construct were counted to assess delivery efficiency.

2.6.3 Image acquisition and processing

Transgenic zebrafish fluorescence images were acquired using a Zeiss AxioZoom.V16 microscope (Carl Zeiss MicroImaging) set with a fluorescence light source (HXP200C), an AxioCam 512 mono (Carl Zeiss MicroImaging) and a PlanNeoFluar Z 1x objective. Image

capture and processing were performed using ZEN 2.5 2012 Blue Edition (Carl Zeiss MicroImaging) software.

2.7 Characterization of Tg(elavl3:GCaMP6f EF05) zebrafish lines using confocal microscopy

2.7.1 Mounting

The selected lines of Tg(elavl3:GCaMP6f EF05) were crossed with a wild-type strain to acquire larvae for confocal imaging. Embryos were collected in an E3 Petri plate and housed in a dedicated incubator overnight. At 1 dpf, the E3 medium was replaced with PTU, and dead embryos were removed. Until the larvae were mounted for confocal imaging, the PTU solution was refreshed daily. At 4 dpf, larvae were screened, and the ones with the strongest fluorescence were chosen for confocal image acquisition at 4 dpf and 6 dpf. Zebrafish larvae were sedated with 0,6mM Tricaine and mounted directly to the glass coverslip (0.17 ± 0.005 mm thick), as follows: in a drop of 1.5 % (w/v) low melting agarose in PBS, larvae were positioned dorsally side up and as straight and close to the coverslip as possible, with the help of forceps. The agarose with the larvae was encircled by a silicone grease well filled with Tricaine. Finally, the microscope slide was placed against the coverslip, forming a watertight seal [113].

2.7.2 Confocal image acquisition and analyses

The images were taken with a Zeiss LSM 710 upright confocal laser scanning microscope, which enables high-resolution optical sectioning of thick fluorescent samples. A long-distance (LD) LCI Plan-Apochromat 25x/0.8 multi-immersion (Zeis) objective was utilized, which is a high-performance lens for deep tissue imaging. A drop of water (refractive index n=1.33) was used as the immersion medium for each slide that was put on the microscope stage. The brain of a zebrafish larva was scanned in stack format. The ZEN 2010 software was used to image the whole brain using a tiling configuration in which two adjacent tiles (one of the forebrain and optic tectum, a second of the cerebellum and hindbrain) with 15 % overlap were collected and patched together. Each tile was imaged at a zoom factor of 0.6x, with a frame size of 566.79X1076.26 µm, 0.8 µm pixel size, and 16-bit depth. The pictures were taken in a stock of

7 pictures with a 1 μm interval and a pixel dwell of 2.27 at 1AU, with the pinhole optimized in the green channel. The open-source program Fiji was used to analyse and treat all the images.

2.8 Fin clipping

Fin clipping was performed on the 2336.2 and 2462.1 (data not shown) fish according to Kosuta C. *et al*, 2018 protocol, adapted to adult zebrafish [114].

2.9 Statistical Analysis

Statistical analysis was carried out on Graph Prism 6 software to determine the embryo survival rate at 24 hpf and efficiency in terms of plasmid delivery of both methods used to generate transgenic zebrafish lines, electroporation, and microinjection, to later compare the two. Delivery success rates were obtained from screening the larvae at 3 to 4 dpf after electroporation or microinjection. All parameters were expressed as means and standard deviation before being used for statistical analysis.

The distribution of the data was assessed for normality using the Shapiro-Wilk test. Normally distributed data were analysed via Student's t-test or one-way ANOVA, and post-hoc comparison was performed using a Tukey test. For non-parametric data, the Mann-Whitney U test or Kruskal-Wallis test were used, with Dunn's test for post-hoc comparisons. For all statistical analyses, p-value was set to 0.05. P-values smaller than 0.05 were considered statistically significant (confidence interval of 95 %). On the data presented in the Results chapter, different letters on the same graph represent significant statistical differences.

2.10 Solutions

Table 2.3- The following is a list of the solutions that were used in this project. The scientific name, abbreviation, composition, and manufacturer are all listed for each solution.

Scientific name	Abbreviation	Composition	Manufacturer
Embryonic medium	E3 (stock solution)	0.25 M NaCl, 8.45 mM KCl, 16.5 mM CaCl2.2H2O and 0,16 M MgSO4.7H2O Filled to 10 L with MilliQ water	-
Embryonic medium	E3 (working solution)	400 mL of E3 stock solution, 60 mL 0.01% Methylene Blue solution Filled to 20 L with system water	-
Methylthioninium chloride	Methylene Blue solution	0.05 g Methylene Blue powder 500 mL MilliQ water	Sigma
Phosphate Buffered Saline	PBS solution	0.13 M NaCl, 2.68 mM KCl, 5.37 mM Na2HPO4.7H2O and 1.76 mM KH2PO4 (pH=7.2)	-
Sodium hypochlorite	Bleach (working solution)	36 ppm 10%	Sigma
Sodium hypochlorite	Bleach (diluted solution)	1%	-
Ethyl 3-aminobenzoate methanesulfonate	Tricaine (stock solution and euthanasia - 15 mM)	2 g Tricaine powder 500 mL reverse osmosis water 10 mL of 1 M Tris (pH= 9)	Sigma
Ethyl 3-aminobenzoate methanesulfonate	Tricaine (working solution- 0.6 mM)	20 mL of Tricaine stock solution 480 mL system water	-
Pronase	Pronase (stock solution- 60 mg/mL)	1 g Pronase 16.7 mL MilliQ water	Roche
Pronase	Pronase (working solution- 6 mg/mL)	100 µL of Pronase stock solution 9.9 mL E3 working solution without methylene blue solution	-
1-phenyl 2-thiourea	PTU (working solution- 0.003%; 200 µM)	30 mg PTU powder 1 L E3 working solution	Sigma
17α,20β-Dihydroxy-4-pregnen-3-one	β-DHP (working solution)	100 nM β-DHP 100 µL absolute etanol 100 mL deionized water	Sigma

3

RESULTS

3.1 Generation of a new neuronal transgenic zebrafish line: elavl3:GCaMP6f EF05

3.1.1 Generation of F-1 populations by microinjection

To generate elavl3:GCaMP6f EF05 transgenics, nacre$^{+/-}$ embryos were injected at the stage of 1 cell with 50 ng/µL of elavl3:GCaMP6f EF05 plasmid DNA and 100 ng/µL of tol2 mRNA to ensure construct integration in the genome. The number of survivors was counted at 24 hpf. Once the larvae were 3-4 days old, they were screened for pan-neuronal expression characteristic of elavl3:GCaMP6f EF05 (unpublished). Table 3.1 summarizes results from injections and screens.

Table 3.1- Injection of elavl3:GCaMP6f EF05 plasmid DNA and Tol2mRNA into 1-cell stage embryos. Number and corresponding percentage of surviving embryos 1 day after birth and injection; of larvae positive for elavl3:GCaMP6f EF05 integration in the genome; and of positive larvae selected to raise. The information available for the 4 lines with an asterisk (*) is limited.

Nº of injected embryos	Nº of screened larvae	Nº of positive larvae	Nº of raised larvae	Survival rate (%) at 24 hpf	Integration rate (%) at 3-4 dpf	Raised rate (%)	Nº of the F-1 line
324	67	0	0	20.7	0.0	0.0	-
224	54	0	0	24.1	0.0	0.0	-
450	86	2	0	19.1	2.3	0.0	-
548	48	1	1	8.8	2.1	100	-
521	97	11	0	18.6	11.3	0.0	-
438	66	9	5	15.1	13.6	55.6	2392
540	110	12	9	20.4	10.9	75.0	2404
469	93	5	0	19.8	5.4	0.0	-
-	-	-	1	-	-	-	5489*
-	-	-	17	-	-	-	5951*
-	-	-	16	-	-	-	6002*
-	-	-	1	-	-	-	6032*

Even though a larger number of embryos was injected, only between 8.8 % to 24.1 % (information taken from the available data) survived until the screen's time point, at 3-4 days old, and even fewer exhibited fluorescence. Seven out of twelve injection rounds were successful for presence of fluorescence in the larvae. Of these, 4 were injected by a colleague and so some information is lacking. Out of the remaining three, in two only a fraction of the positive larvae was raised (55.6 % and 75 %). The remaining had developmental malformation and were euthanized. Healthy larvae, exhibiting a widespread bright green fluorescence, were raised to adulthood, as F-1 lines designated with the numbers indicated on Table 3.1. (2392, 2404, 5489, 5951, 6002 and 6032).

3.1.2 Line establishment

3.1.2.1 Assessment of germline transmission

Once F-1 fish reached sexual maturity, they were individually crossed, and progeny screened for germline transmission of the injected elavl3:GCaMP6f EF05 construct (Table 3.2).

Table 3.2- Screen results of the F-1 lines raised. Number and corresponding percentage of F-1 fish with germline transmission; average percentage of positive progeny (F0); description of expression pattern and fluorescence intensity of progeny (F0); identification and number of fish and lines of F0 raised.

F-1 line	Nº of F-1 fish screened	Nº (%) of F-1 fish with germline transmission	Mean % F0 positive larvae	Expression pattern	Fluorescence intensity	Nº of F0 lines raised	Nº of F0 larvae raised	Nº of F0 lines
5489	1	1 (100 %)	2.8	pan-neuronal in all positive larvae	Very dim	1	3	2335
5951	14	14 (100 %)	16	pan-neuronal in all positive larvae	Moderate to very bright within each F0 progeny	20 (from 13 F-1 fish)	331	2461 2462 2552 .....
6002	8	1 (12.5 %)	1.40	pan-neuronal in all positive larvae	Dim	1	6	2334
6032	1	1 (100 %)	4.2	pan-neuronal in all positive larvae	Moderate	1	4	2336
Total	24	17 (70.83 %)	9.86	-	-	23	344	-

The efficiency of the injections in terms of germline transmission of elavl3:GCaMP6f EF05 varied a lot, from 12.5 % to 100 % of the fish raised after injection round passing the transgene to the progeny, but in most lines, it was below 28 % (data not shown). The overall mean percentage of integration among the progeny was 9.86 %. Interestingly, the expression pattern on the positive larvae looked the same for all lines. What varied among the progeny of F-1 individuals was the fluorescence intensity. In addition, in the F0 clutches from 5951 individuals, the level of fluorescence intensity varied, whereas for each clutch from lines 5489, 6002 and 6032, all positive larvae looked similar.

3.1.2.2 A promising F-1 line

Out of the six F-1 populations raised after injections, fish from line 5951 stood out because all of them had germline transmission. Line 5951 was also the only in which the percentage of positive progeny was greatly higher, 16% of the larvae showed bright green, fluorescent pan-neuronal expression (Figure 3.1). A strong cardiac expression was also observed but corresponded to a green cardiac marker present on the isl2b:Gal4$^{+/-}$ embryos used for microinjection that produced line 5951, so it was ignored. Line 6032 also had 100 % positives but was not considered as successful because it corresponded to only one fish.

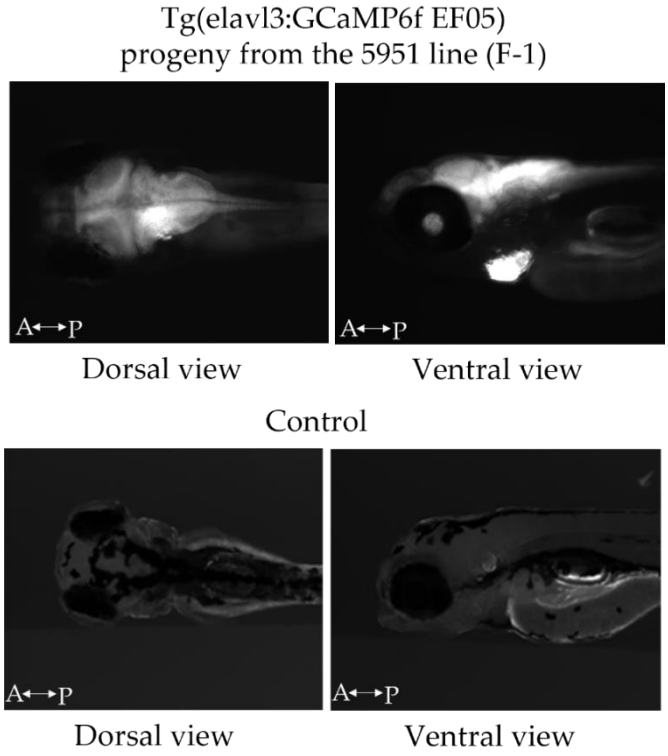

Figure 3.1- Stable expression of elavl3:GCaMP6f EF05 construct in the 5951 F-1 line progeny. Offspring larvae from the F-1 line were screened at 3-4 dpf. On top: Tg(elavl3:GCaMP6f EF05) larvae with pan-neuronal expression pattern; On the bottom: wild-type control. A: anterior; P: posterior.

Taken all the above into consideration, line 5951 was selected for further steps towards the establishment of a Tg(elavl3:GCaMP6f EF05) stable line. Because offspring of all 5951 individuals showed a bright homogeneous fluorescence, all fish were used for raising founder (F0) populations using the positive larvae from each clutch.

3.1.2.3 Founders (F0) selection and establishment of Tg(elavl3:GCaMP6f EF05) line (F1)

When F0 populations derived from 5951 F-1 were adults, fish were individually outcrossed, and progeny screened for confirmation of stable germline transmission. Out of the 31 F0 fish from all the 12 lines screened, 6 were selected as founders for the 7 F1 lines generated (one of the founders generated two F1 lines), based on the presence of the expected complete pan-neuronal expression pattern with high fluorescence intensity on the positive offspring, observed under the Zeiss AxioZoom stereoscope (Figure 3.2). The selection of larvae to be raised into the F1 generation was random within the positive larvae. To confirm if the established lines agreed with the needs of the research collaborator that was going to use the line after being generated, for functional visuomotor behavioural studies, some 3-4 dpf larvae from 3 different founders were screened by that researcher using light-sheet microscopy.

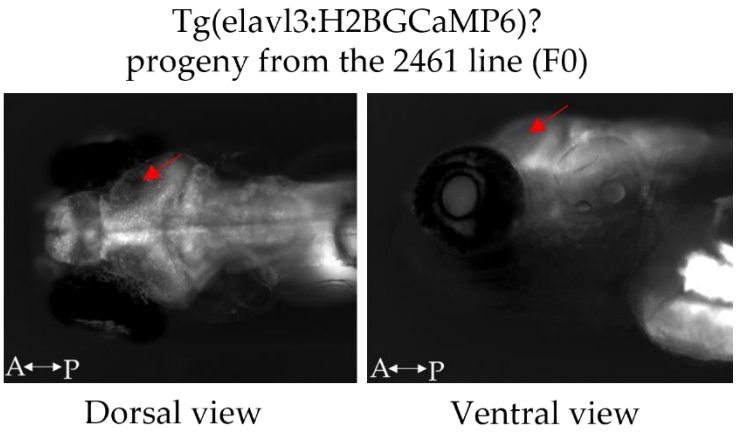

Figure 3.2- F1 larvae from 2461.9 founder. Tg(elavl3:H2BGCaMP) zebrafish larva with a close to complete pan-neuronal expression pattern screened at 3-4 dpf. Red arrows pointed at the optic tectum where almost no expression can be seen. A: anterior; P: posterior.

3.1.2.4 Genetically contaminated line

The results of light-sheet imaging (not shown) revealed that the 3 F1 clutches, derived from 3 different founder fish, had a nuclear-localized pan-neuronal expression pattern, contrary to the expected cytoplasmic elavl3:GCaMP6f EF05. So far, all that was known was that: 1) the construct had a GCaMP indicator expressed under the control of the elavl3 promotor; 2) it had a nuclear-localized pan-neuronal expression pattern; and 3) it had a high green fluorescence intensity, indicating that it came from an established line. It could either be elavl3:H2BGCaMP6s or elavl3:H2BGCaMP6f, the only 2 lines in the facility that respected the above criteria. To confirm this, a caudal fin sample from each of the 6 founders was collected and sent for sequencing against H2B (that promotes nuclear expression) and against EF05. Sequencing revealed that EF05 was not present and that H2B was. In conclusion, the integrated

construct in the transgenic line was found to be one of the 2 nuclear elavl3:H2BGCAMP lines (6s or 6f).

A second careful analysis of the F1 larvae expression confirmed that expression appeared spotted, typical of nuclear expression (Figure 3.2), rather than a more homogeneous expression characteristic of a cytoplasmic pan-neuronal expression pattern (Figure 3.4). Moreover, the fluorescence was almost absent in the optic tectum.

3.1.2.5 Finding the source of contamination

It was crucial to figure out where this contamination originated from because it could jeopardise all the work developed up until this point. Unexpectedly, around the same time a collaborator identified a Tg(isl2b:Gal4$^{+/+}$) line that had been genetically compromised. The contamination in this line had a pan-neuronal expression pattern and a nuclear-localized bright green fluorescence (Figure 3.3), similar to that of 5951 (F-1) and 2461 (F0) lines: spotted expression pattern and absence of expression in the optic tectum. By checking the identification of the Tg(isl2b:Gal4$^{+/+}$) line used for generating embryos for the injections that originated the lines now proved contaminated, it was confirmed that it was indeed the same Tg(isl2b:Gal4$^{+/+}$) line reported as contaminated by a colleague. These embryos were grown to adulthood becoming the 5951 F-1 line. As a result, all F0 fish descended from F-1 line 5951 had been genetically compromised, and so, the establishment of these lines ceased.

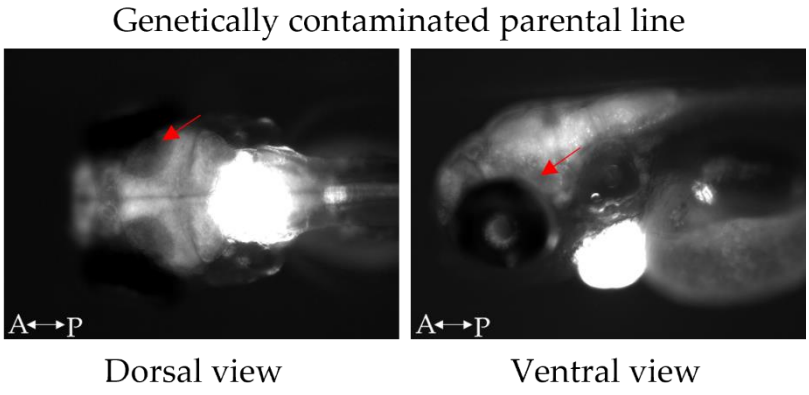

Figure 3.3- Expression pattern of the Tg(isl2b:Gal4$^{+/+}$) genetically contaminated line. Zebrafish larva with a nuclear-localized pan-neuronal expression pattern screened at 3-4 dpf. Red arrows pointed at the optic tectum where almost no expression can be seen. A: anterior; P: posterior.

3.1.2.6 Selection of new F-1 fish for line establishment

To find a new injected fish with germline transmission of the correct construct, the results from the screens of the remaining 3 (5489, 6002 and 6032 lines) F-1 populations were

analysed again (Table 3.2). The most promising line out of the other two F-1 lines available was 6032. Despite consisting of a single fish, a new screen confirmed that its progeny exhibited, at 3-4 dpf, a homogenous expression pattern in all nerve cells, including the optic tectum, accompanied by a moderate rather than high green fluorescence intensity, more typical of GCaMP6fEF05 and cytoplasmic expression (Figure 3.4). In addition, the fish 6032 produced a low percentage of integration in the progeny, with an average of 4.2 %. Such low germline integration efficiency agreed with what was expected for large constructs such as elavl3:GCaMP6f EF05 (~10kb), because these are more difficult to integrate into the genome than small inserts. Besides, the lower the number of positive larvae, the higher the chances of construct insertion on a single genomic site. Considering all the above, fish 6062 was selected and the 4 larvae, among the progeny, that had complete pan-neuronal expression and highest fluorescence intensity, were raised until adulthood, as the new founders, named 2336.1 to 2336.4.

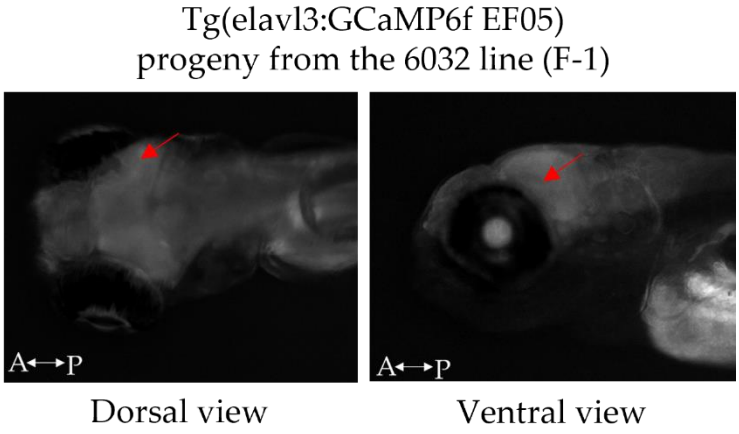

Figure 3.4- Expression pattern of the Tg(elavl3:GCaMP6f EF05) 6032 line progeny. Transgenic zebrafish larva with a complete pan-neuronal expression pattern screened at 3-4 dpf. Red arrows pointed at the optic tectum represent where fluorescence was observed for a cytoplasmic marker. A: anterior; P: posterior.

3.1.2.7 Final establishment of Tg(elavl3:GCaMP6f EF05) fish line

When the 2336 founders were adults, they were outcrossed with a wild-type strain to evaluate whether there was germline transmission (Table 3.3).

Table 3.3- Screen results of the F0 lines raised from the 6032 fish. Number and corresponding percentage of F0 fish with germline transmission; average percentage of positive progeny (F1); description of expression pattern and fluorescence intensity of progeny (F1); identification and number of fish and lines of F1 raised.

F0 line and nº of each fish	Nº of F0 fish screened	Nº (%) of F0 fish with germline transmission	Mean % F1 positive larvae	Expression pattern	Fluorescence intensity	Nº of F1 lines raised	Nº of F1 larvae raised	Nº of F1 lines
2336.2	1	1 (100 %)	20.9	pan-neuronal in all positive larvae	Very bright	1	36	2830
2336.3	1	1 (100 %)	34	pan-neuronal in all positive larvae	Dim to very bright	2	55	2863 2887
2336.4	1	1 (100 %)	31.3	pan-neuronal in all positive larvae	Bright	1	22	2886
Total	3	3 (100 %)	28.5	-	-	4	113	-

The efficiency of germline transmission was constant through all the F0 fish, however this parameter cannot be taken into account as it was in previous screens due to the fact that it is the representation of only one fish. The overall mean percentage of integration among the progeny was 28.5 % and all the fish stood more or less within proximity of that value. All positive larvae from all of the 3 2336 fish were characterized by a complete pan-neuronal expression pattern, illustrated in Figure 3.5. What varied among the progeny of F0 individuals was the fluorescence intensity. In addition, in the F1 clutches from the 2336.3 fish, the level of fluorescence intensity varied, whereas for each clutch from fish 2336.2 and 2336.4, all positive larvae looked similar.

A sample of the caudal fin from a 2336 founder was collected and sequenced, and the results supported screen findings, revealing that the integrated construct in this new line establishment was the elavl3:GCaMP6f EF05 construct.

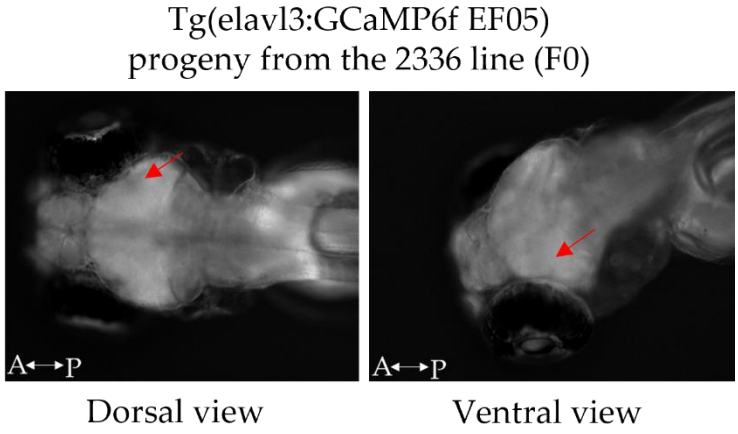

Figure 3.5- Expression pattern of the Tg(elavl3:GCaMP6f EF05) 2336 line progeny. Transgenic zebrafish larva with a complete pan-neuronal expression pattern screened at 3-4 dpf. Red arrows pointed at the optic tectum represent where fluorescence was observed for a cytoplasmic marker. A: anterior; P: posterior.

In addition, these findings were supported by light-sheet imaging (data not shown), carried out by the same collaborator. Thus, the larvae with the highest fluorescence intensity and a complete pan-neuronal expression pattern were chosen to generate F1 populations and complete the establishment of new transgenic lines for the injected construct.

Once reached sexual majority, the adult transgenic zebrafish from the raised F1 line, the 2830 line, were outcrossed with a wild-type strain. After reaching 3-4 days old the larvae were pre-screened for pan-neuronal expression and taken to the confocal microscope for image acquisition.

3.1.2.8 Fine characterization of the Tg(elavl3:GCaMP6f EF05) lines using confocal microscopy

Confocal microscopy was used to have a more detailed characterisation of the expression pattern of the generated new lines and to ensure the presence of a pan-neuronal line with cytoplasmic expression. The two larvae with best expression on Zeiss AxioZoom stereoscope were mounted and imaged on Zeiss confocal at 4 dpf (Figure 3.6). The newly established Tg(elavl3:GCaMP6f EF05) 2830 line displayed the characteristic elavl3 pan-neuronal expression pattern exhibiting a cytoplasmic-localized fluorescence in all nerve cells. In this line, contrary to what was observed in the first line establishment attempt, the optic tectum (TeO) area exhibited fluorescence. These results along with the ones from sequencing confirmed the correct transgenic line establishment.

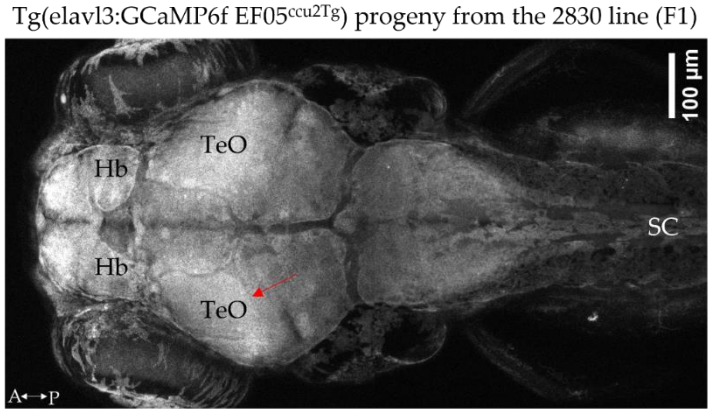

Figure 3.6- Expression pattern of the Tg (elavl3:GCaMP6f EF05) F1 line progeny. Confocal microscope image of dorsal view of a transgenic zebrafish larva at 4 dpf. Tg (elavl3:GCaMP6f EF05) exhibits an elavl3 expression pattern. Red arrow pointed at the optic tectum represents where fluorescence was observed for a cytoplasmic marker. Hb: habenula; SC: spinal cord; TeO: optic tectum. A: Anterior; P: Posterior.

3.2 Optimizations to the electroporation protocol in zebrafish embryos

This project was designed at a time when electroporation protocols available in the literature for transgenesis in zebrafish were rarely reported before. However, in 2020, soon before the beginning of the project, Zhang, C. and colleagues published their work on "Transgenic Expression and Genome Editing by Electroporation of Zebrafish Embryos" [112]. In our hands, the replication of the published protocol resulted in poor survival and absence of successful construct delivery, and therefore, it was used as a base on the top of which the current project aimed at developing several optimizations.

The 10xUAS:LSSmOrange construct was chosen to conduct the electroporation protocol optimizations because of being short (~2.7 kb) and easy to deliver, and because it's expression could be evaluated based on a well characterized promoter line, Tg(isl2b:Gal4). The induction of transient expression does not require the addiction of tol2 transposase mRNA to the injection mix as it is not necessary for the plasmid to integrate into the genome. Transient expression was used to test the efficacy of electroporation protocol optimizations.

3.2.1 Dechorionation method

The first optimization was the method for dechorionation, or chorion removal, the technique that precedes electroporation to boost the chances of integration. Two distinct methods of dechorionation were tested and compared in terms of embryo survival: manual

dechorionation as a mechanical method, and enzymatic method utilizing incubation in pronase, which is an enzyme used to digest the zebrafish embryos chorion (external membrane) (Figure 3.7).

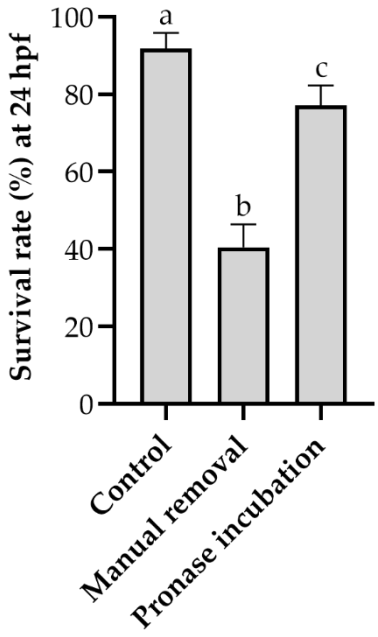

Figure 3.7- Survival rates (mean ± standard deviation error) at 24 hpf, following manual and enzymatic chorion removal of 8-cell stage zebrafish embryos. Non-manipulated embryos were used as control. Different letters (a, b and c) indicate statistically significant differences compared to all other groups. All conditions were tested seven times (n=7) each time using 15 embryos. A Tukey's multiple comparison test was performed with significance set at p-value < 0.05.

The survival rate of the non-dechorionated control (91.77 % ± 4.08) was significantly higher than after both dechorionation methods. Between dechorionation methods, survival was higher when the dechorionation method applied was pronase incubation (77.14 % ± 5.19) compared to when the chorions were manually removed (40.43 % ± 5.94). For this reason, pronase incubation was selected as the dechorionation method of choice for all subsequent electroporation experiments.

The effect of agitation during the pronase incubation of 8-cell stage zebrafish embryos on survival at 24 hpf was next assessed. It can be observed in Figure 3.8 that agitation does not affect the embryo's survival, as there is no significant difference in survival when under agitation (75.10 % ± 4.41) compared to its absence (77.43 % ± 5.16); However, there is a significant difference of these two experimental groups when compared to the survival retrieved by the control group (89.11 % ± 6.57). Hence, agitation is not necessary for an

effective dechorionation process. As a result, agitation was not included in the optimized protocol developed in this thesis.

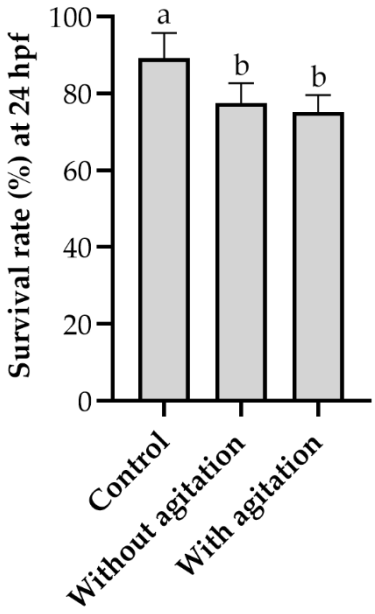

Figure 3.8- Survival rates (mean ± standard deviation error) at 24 hpf, following enzymatic chorion removal of 8-cell stage zebrafish embryos with and without agitation. Non-manipulated embryos were used as control. Different letters (a and b) indicate statistically significant differences compared to all other groups. All conditions were tested seven times (n=7) each time using 15 embryos. A Tukey's multiple comparison test was performed with significance set at p-value < 0.05.

3.2.2 Number of embryos in the electroporation chamber

The optimized number of embryos to carry out the electroporation was 30. This number was chosen considering that (1) embryos could not touch the electrodes and (2) embryos had to be displayed in a non-compact manner to ensure that all embryos would experience the same applied pulses, in intensity. The 30 embryos were aligned in a row perpendicularly to the applied pulses as exemplified in Figure 3.9. All posterior optimizations were done electroporating 30 embryos in each cycle of experiments.

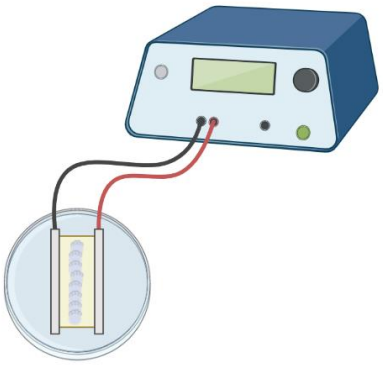

Figure 3.9- Representation of embryo display in the electroporation chamber throughout optimizations to the electroporation protocol. Created in BioRender.com

3.2.3 Volume of the DNA solution in the electroporation chamber

The electroporation chamber used in this work was custom-made to fit more embryos than the commercially available chamber. For this reason, there was the need to adjust the volume of the electroporation mix. The minimal volume of DNA solution found to be required for the electroporation procedure was 150 µL. This value represents a compromise between ensuring that all embryo's surface is covered while using the minimal amount of DNA possible.

3.2.4 Developmental stage used for electroporation

Employing the optimizations stated in the previous section, electroporation was tested applying the highest set of electrical parameters assessed by Zhang, C., *et al.* 2020 as a starting point [112]. For this, 30 zebrafish embryos at 8-cell stage were electroporated with plasmid DNA at 180 ng/µL (concentration of the working stock) using poring pulses of 50 V voltage, 5 ms pulse length, 50 ms pulse interval, 4 pulses, 10 % voltage decay, and + polarity orientation, followed by transfer pulses of 10 V voltage, 50 ms pulse, 50 ms pulse interval, 5 pulses, 40 % voltage decay, and ± polarity orientation.

There were no positive embryos out of the 210 embryos tested. As a result, not only was there no integration, but the mortality rate was also high, over 85% of mortality, indicating that there was room for further optimizations. Therefore, the first thing we decided to test next was the developmental stage of the electroporated embryos, as 8-cell stage is not ideal for generating stable transgenic lines. For that reason, the same experiment was carried out using 1-cell stage embryos, and survival rate of the electroporated embryos was assessed and compared at each step of the protocol (Figure 3.10).

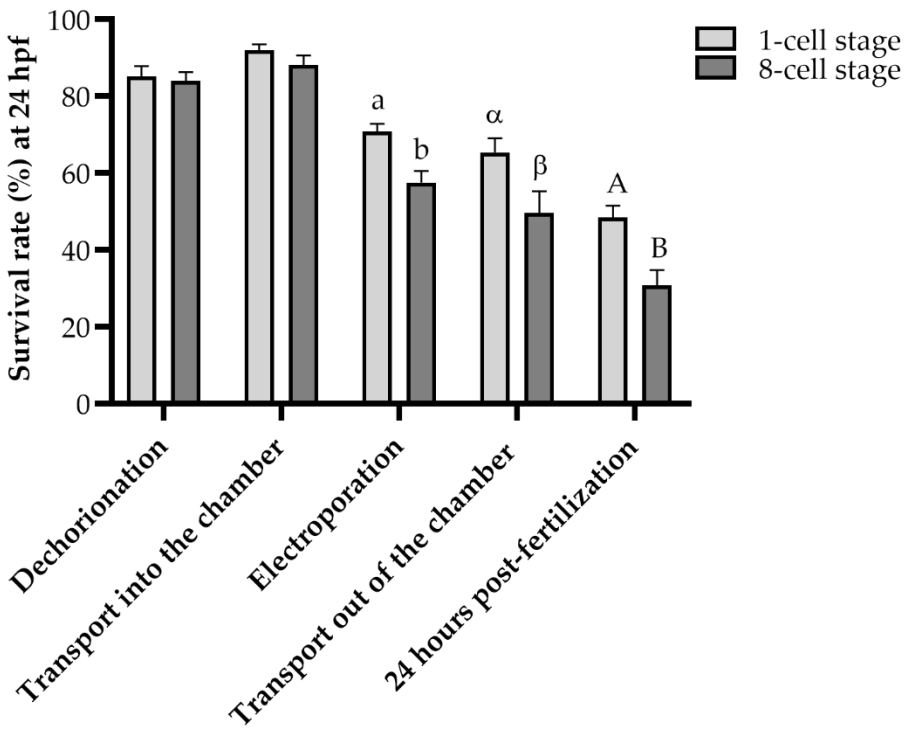

Figure 3.10- Survival rates (mean ± standard deviation error) at 24 hpf, following each step of the electroporation protocol of 8- and 1-cell stage zebrafish embryos. Different letters indicate statistically significant differences compared to all other groups. Different sets of letters (a,b; A,B and α,β) refers to comparisons within a different cell stage. All conditions were tested seven times (n=7) each time using 30 embryos. A Tukey's multiple comparison test was performed with significance set at p-value < 0.05.

The survival rate after dechorionation was not affected by the embryo's developmental stage, as no significant difference was found when comparing the survival of 1-cell stage (85.09 % ± 2.77) to 8-cell stage embryos (83.99 % ± 2.33) after that procedure. Handling and transport of the dechorionated embryos into the electroporation chamber also did not seem to affect survival when handling 1-cell stage embryos (91.93 % ± 1.63) compared to when 8-cell stage embryos were handled (88.17 % ± 2.43). Contrarily, when 1-cell stage embryos were electroporated, the survival was significantly higher (70.80 % ± 2.09) than when 8-cell stage embryos were electroporated (57.56 % ± 2.95). A significantly higher survival was also observed during the transport of embryos from the electroporation chamber to a petri dish where they were incubated: survival rate of 65.40 % ± 3.69 for 1-cell stage embryos versus 49.66 % ± 5.60 for 8-cell stage embryos. Lastly, on the day after the electroporation, the survival rate was assessed again and, once more, the embryos electroporated at the 1-cell stage presented a significantly superior survival rate (48.49 % ± 3.04), when compared to the electroporated 8-cell stage embryos (30.77 % ± 3.99).

The data in the Figure 3.10 was normalized, which means that the survival rate of each step of the procedure, for both 1- and 8-cell stage, was calculated considering the number of embryos alive in the beginning of each step, and not the 30 embryos used as starting point for all electroporation protocol. For that reason, it was also possible to retrieve information concerning the steps of the process that had the greatest impact on embryo survival.

Considering each step of the protocol individually, the lowest survival rates were obtained upon and after the electroporation step, indicating that electroporation itself is not only a critical step that needs optimizations, but also pivotal for the survival rate at the end of the whole electroporation protocol.

The overall survival after the entire electroporation protocol, the accumulative effect of every step on the procedure, was also evaluated, and the findings are shown in Figure 3.11.

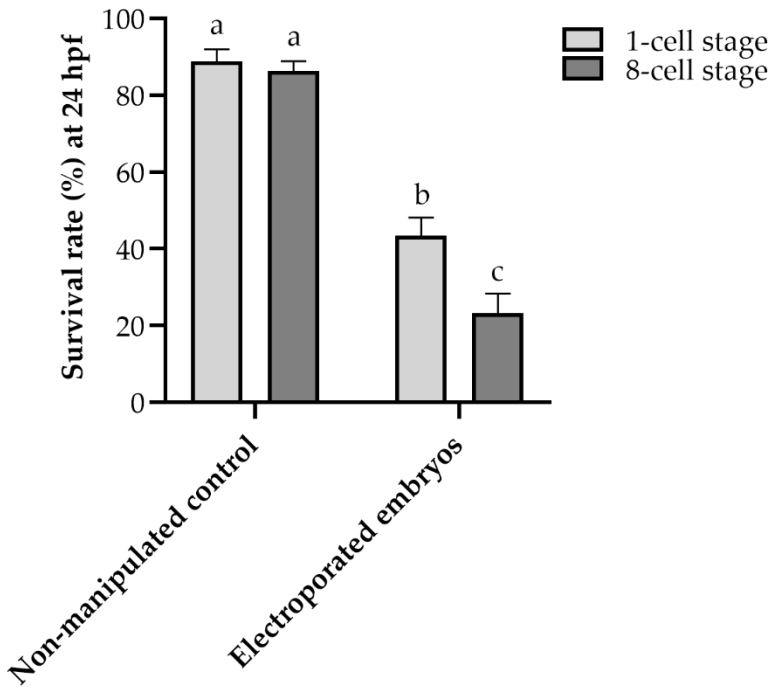

Figure 3.11- Survival rates (mean ± standard deviation error) at 24 hpf, following the entire electroporation procedure of 8- and 1-cell stage zebrafish embryos. Non-manipulated embryos were used as control. Different letters (a, b and c) indicate statistically significant differences compared to all other groups. All conditions were tested seven times (n=7) each time using 30 embryos. A Tukey's multiple comparison test was performed with significance set at p-value < 0.05.

The experiment using 1-cell stage embryos yielded a significantly higher survival rate (43.47 % ± 4.64) when compared to an experiment using 8-cell stage embryos (23.33 % ± 5.09). Although the use of 1-cell stage embryos allowed the survival rate to almost duplicate (1.86 times higher), it was still significantly lower than the corresponding non-manipulated control

(88.89 % ± 3.10). Following these results, further optimizations to the electroporation protocol were performed only in 1-cell stage embryos.

However, the cell stage optimization to the electroporation protocol, using 1-cell stage embryos instead of 8-cell stage embryos, still yielded an overall survival rate of less than 45 percent. For that reason, it was clear that further optimizations of the electroporation protocol were still required, so we proceeded with further tests.

3.2.5 Electrical pulses

The electrical parameters involved in electroporation are determinant both for the efficiency of molecules delivery and for the survival of the embryos. As it will be demonstrated in this work, higher voltages lead to a decrease in survival, while lower voltages lead to inefficiency of the process. As a result, it was necessary to find a set of pulses that maximized the probability of the construct integrating without compromising the embryos' survival. To accomplish so, it was essential to first identify the upper maximum limit, above which the technique began to cause more harm than good. After identifying this value, the pulse was gradually reduced until a value was obtained that allowed the integration rate and survival rate to be balanced. According to Zhang, C. and colleagues, the typical transfer pulse can be one of two values: 5 V and 10 V. For a first approach the transfer pulse was set at 10 V and different intensities of the poring pulse were tested, always electroporating the 10X:UAS:LSSmOrange plasmid at a concentration of 180 ng/μL. The results can be found in Figure 3.12 below [112].

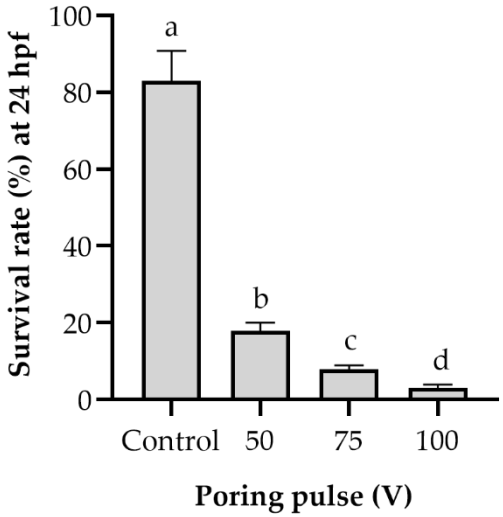

Figure 3.12- Survival rates (mean ± standard deviation error) at 24 hpf, following electroporation of 1-cell stage zebrafish embryos using different poring pulse voltages with a fixed transfer pulse of 10 V, using a 10xUAS:LSSmOrange plasmid DNA concentration of 180 ng/μL. Non-manipulated embryos were used as control.

Different letters (a, b, c and d) indicate statistically significant differences compared to all other groups. All conditions were tested seven times (n=7) each time using 30 embryos. A Tukey's multiple comparison test was performed with significance set at p-value < 0.05.

Under these conditions, no positive embryos for 10xUAS:LSSmOrange were obtained. There was a considerable decline in the survival rate when the transfer pulse was fixed at 10 V and the poring pulse was gradually raised. The lowest poring pulse tested, 50 V, was shown to be the most effective in terms of survival (17.90 % ± 2.13), while the highest poring pulse tested, 100 V, resulted in a significant reduction in survival (3.00 % ± 0.94). When the transfer pulse is held at 10 V, a generalized poor survival can be seen when compared to the unmanipulated control (83.02 % ± 7.76). This raised the possibility that better results could be obtained if, instead of 10 V, 5 V of transfer pulse was used to test the two most promising poring values, according to the data. For that purpose, a comparison was conducted in which 50 V and 75 V of poring pulse were tested against 10 V and 5 V of transfer pulse, with a DNA concentration of 180 ng/μL, graphically displayed in Figure 3.13.

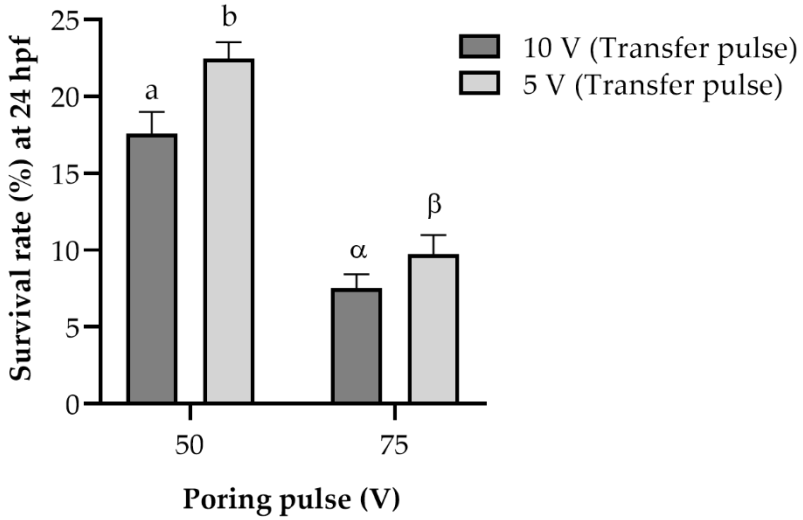

Figure 3.13- Survival rates (mean ± standard deviation error) at 24 hpf, following electroporation of 1-cell stage zebrafish embryos using different combinations of poring and transfer pulse voltages, using a 10xUAS:LSSmOrange plasmid DNA concentration of 180 ng/μL. Non-manipulated embryos were used as control. Different letters indicate statistically significant differences compared to all other groups. Different sets of letters (a,b and α,β) refers to comparisons within a different transfer pulse voltage. All conditions were tested seven times (n=7) each time using 30 embryos. A Tukey's multiple comparison test was performed with significance set at p-value < 0.05.

When the poring pulse was set on 50 V, embryo survival was substantially higher when the transfer pulse was 5 V (22.44 % ± 1.10) than when it was 10 V (17.58 % ± 1.40). The same is true when the poring pulse was defined has 75 V: survival was significantly higher when the transfer pulse was 5 V (9.74 % ± 1.25) versus 10 V (7.50 % ± 0.93). Thus, a 5 V transfer pulse

favours survival compared to a 10 V transfer pulse, regardless of the poring pulse employed. Surprisingly, the same holds true when the poring pulse was 50 V instead of 75 V. Specifically while employing a 5 V transfer pulse, embryo survival was considerably higher when the poring pulse was 50 V rather than 75 V.

Similarly, when the transfer pulse was set at 10 V, the survival rate of the poring pulse at 50 V was higher than the pulse set at 75 V. According to these results, the transfer pulse at 5 V had the highest survival rates regardless of the value of the poring pulse. In addition, the lowest poring pulse tested (50 V), always retrieved the greatest survival rate, regardless of the value of the transfer pulse.

Setting the transfer pulse at 5 V had a positive impact on embryos' survival, and this impact was greater the lower the voltage of the poring pulse employed. Despite having a higher survival rate than when the pulse was set at 75 V, the 50 V poring pulse still had a relatively low overall survival rate. To enhance the survival rate, lower poring pulses were tested while keeping the transfer pulse at 5 V and the DNA concentration at 180 ng/μL, the results of which are presented in Figure 3.14.

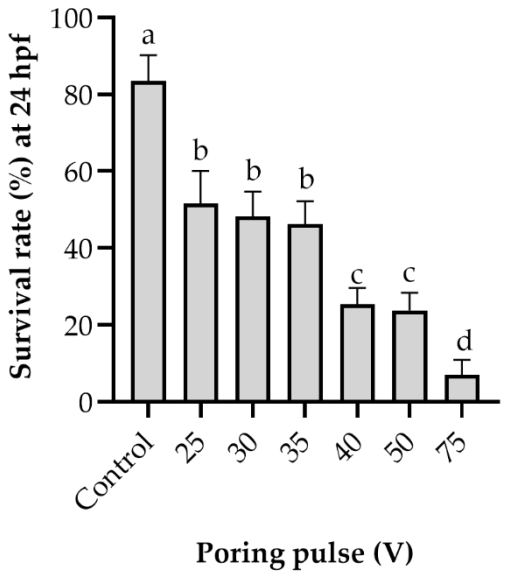

Figure 3.14- Survival rates (mean ± standard deviation error) at 24 hpf, following electroporation of 1-cell stage zebrafish embryos using different poring pulse voltages with a fixed transfer pulse of 5 V, using a 10xUAS:LSSmOrange plasmid DNA concentration of 180 ng/μL. Non-manipulated embryos were used as control. Different letters (a, b, c and d) indicate statistically significant differences compared to all other groups. All conditions

were tested seven times (n=7) each time using 30 embryos. A Tukey's multiple comparison test was performed with significance set at p-value < 0.05.

There were no significant differences in embryo survival between the 25, 30, and 35 V poring pulses (51.66 % ± 8.36, 48.31 % ± 6.41 and 46.29 % ± 5.91 respectively) for the transfer pulse set at 5 V. However, these three different poring pulses exhibited a significant difference in embryo survival when compared to the control (83.48 % ± 6.76) as well as all other poring pulse values tested. These three poring pulses had the highest survival rates of all the poring voltages tested.

When embryos were exposed to a 40 V poring pulse (25.36 % ± 4.23) versus a 50 V pulse (23.76 % ± 4.60), no significant differences in survival rate were found. However, these two poring voltages differ significantly from the control (83.48 % ± 6.76) and the remainder of the voltages under research.

The poring pulse with the lowest survival rate was 75 V (6.96 % ± 4.02), confirming previous observations in this work. When compared to the control and all other voltages studied, significant differences were observed. It was confirmed that this high pulse voltage is unsustainable for the embryos' survival.

3.2.6 DNA dilution medium

Until now, a DNA concentration of 180 ng/μL has been used because it was the working stock concentration already available in the laboratory and was close enough to the plasmid concentration used by Zhang, C., *et al.* 2020 [112]. However, the working stock quickly ran out, and a new working stock had to be made from more concentrated stock. As a result, by measuring the impedance, it was necessary to determine which dilution medium would be the most appropriate. The three media available in the research facility were tested for this (Figure 3.15).

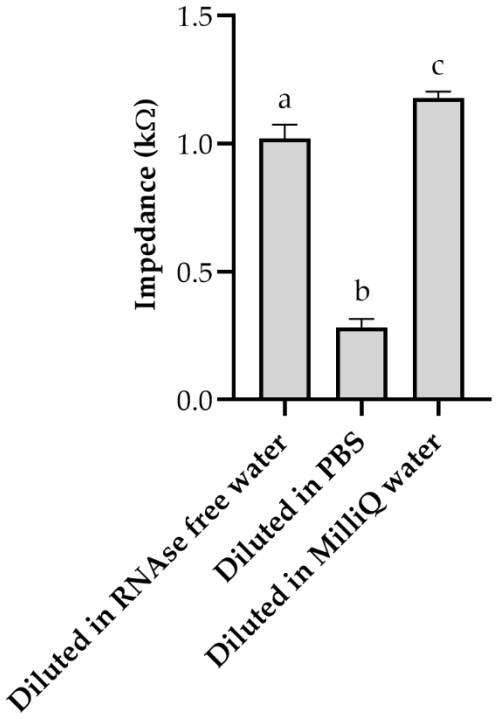

Figure 3.15- Impedance values (mean ± standard deviation error), following different DNA dilution mediums: RNAse free water; Phosphate Buffered Saline (PBS); and MilliQ water using a poring pulse of 50 V and a transfer pulse of 5 V. The 10xUAS:LSSmOrange plasmid DNA was used at a concentration of 180 ng/μL. Different letters (a, b and c) indicate statistically significant differences compared to all other groups. All conditions were tested four times (n=4) each time using 30 embryos. A Tukey's multiple comparison test was performed with significance set at p-value < 0.05.

PBS (0.28 % ± 0.03) was the dilution medium that yielded the lowest impedance value, followed by RNAse free water (1.02 % ± 0.05) and MilliQ water (1.18 % ± 0.03). In regard to impedance values, the three dilution mediums were significantly different from one another. The chosen dilution medium was the one that retrieved the lowest impedance values; hence PBS was chosen for further DNA dilutions.

3.2.7 DNA concentration

Because the current working stock had just run out, dilution mediums were tested to make new working stock from more concentrated stock. It created an excellent opportunity to test novel concentrations. The survival and integration rates for a variety of DNA concentrations were assessed to determine which DNA concentration was the most suited to be used in the working stock for further electroporation protocol optimizations (Figure 3.16).

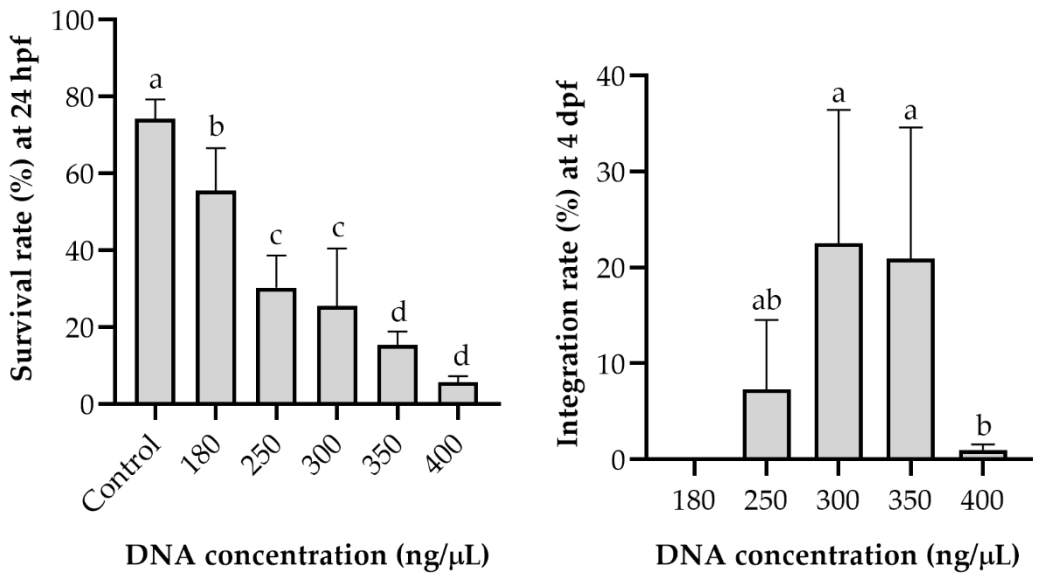

Figure 3.16- Left: Survival rates (mean ± standard deviation error) at 24 hpf, following electroporation at different 10xUAS:LSSmOrange plasmid DNA concentrations of 1-cell stage zebrafish embryos using a poring pulse of 50 V and a transfer pulse of 5 V; Right: Integration rates (mean ± standard deviation error) at 4 dpf, following electroporation at different 10xUAS:LSSmOrange plasmid DNA concentrations of 1-cell stage zebrafish embryos. Non-manipulated embryos were used as control. Different letters (a, b, c and d) indicate statistically significant differences compared to all other groups. All conditions were tested fourteen times (n=14) each time using 30 embryos. A Tukey's multiple comparison test (left) and a Dunn's test (right) were performed with significance set at p-value < 0.05.

The 180 ng/μL was, in fact, the DNA concentration value that yielded the best survival rates (55.53 % ± 11.03). It was the number that was closest to the control, although it was significantly different from the control (74.09 % ± 5.13) and all other concentrations tested. Then there were the 250 and 300 ng/μL concentrations (30.24 % ± 8.43 and 25.46 % ± 15.00 respectively), which, while there were no differences in survival between these two concentrations, were considerably different not only from the control but also from all other conditions under study. Finally, the lowest survival values were found between 350 and 400 ng/μL (15.27 % ± 3.55 and 5.66 % ± 1.52 respectively), which were clearly harmful concentrations for the great majority of embryos. In general, the ideal DNA concentration levels to implement in terms of survival would have been 180, 250, and 300 ng/μL.

In terms of integration, the concentration at 180 ng/μL did not exhibit any, matching the results acquired so far. This may have had a part in the lack of results to this point. The 400 ng/μL had the lowest integration rate (0.99 % ± 0.57), which is significantly lower than any other concentration at which integration has been recorded. The 250 ng/μL had an intermedia integration rate (7.33 % ± 7.20) that was not significantly different from any other

concentration tested. Although the mean value was quite different from other values, the standard deviation values implied that all those concentrations could present the same mean. It would be incorrect to say that the integration rates for those concentrations are statistically different. Finally, the concentrations of 300 and 350 ng/μL showed the greatest outcomes in terms of integration rate (22.53 % ± 13.89 and 20.91 % ± 13.66 respectively). Despite their unpredictability due to standard deviation intervals, these were the most promising concentrations tested.

In conclusion, the concentration of 300 ng/μL exhibited a good integration rate and a substantial survival rate. It allowed for a fair chance of integration without jeopardizing the embryo's survival, and so, it was the chosen working stock concentration from then on.

3.2.8 Poring pulse

By changing the DNA dilution medium and concentration, it was essential to re-optimize the electrical pulses now that the circumstances had changed in such a way that integration was observed for the first time in this research. The poring pulse was adjusted for this, employing a transfer pulse of 5 V, previously reported as the optimum voltage for the transfer pulse; the results are plotted in Figure 3.17.

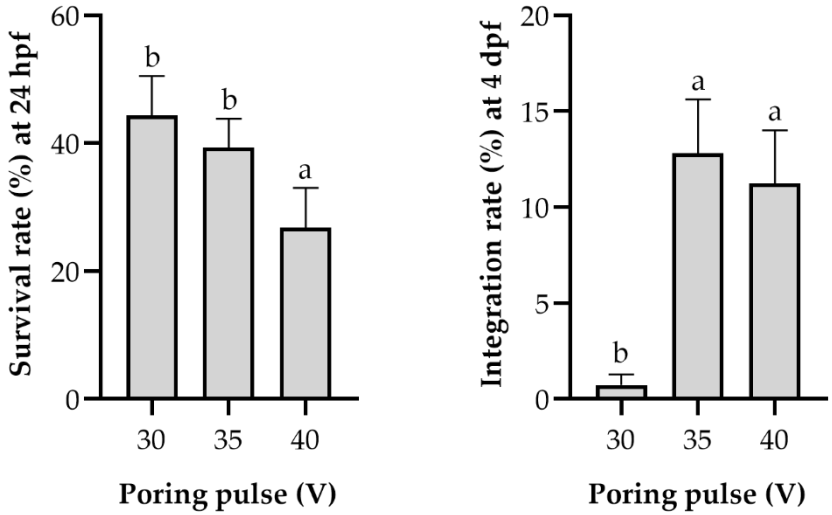

Figure 3.17- Left: Survival rates (mean ± standard deviation error) at 24 hpf, following electroporation of 1-cell stage zebrafish embryos using different poring pulse voltages with a fixed transfer pulse of 5 V, using a 10xUAS:LSSmOrange plasmid DNA concentration of 300 ng/μL; Right: Integration rates (mean ± standard deviation error) at 4 dpf, following electroporation using different poring pulse voltages with a fixed transfer pulse of 5 V of 1-cell stage zebrafish embryos, using a 10xUAS:LSSmOrange plasmid DNA concentration of 300 ng/μL. Non-manipulated embryos were used as control. Different letters (a and b) indicate statistically significant differences

compared to all other groups. All conditions were tested seven times (n=7) each time using 30 embryos. A Tukey's multiple comparison test (left) and a Dunn's test (right) were performed with significance set at p-value < 0.05.

In terms of survival, the poring pulses with the best outcome were the 35 and 30 V (39.36 % ± 4.46 and 44.31 % ± 6.20 respectively). These two voltages did not differ significantly from one another but did when compared to a 40 V pulse (26.81 % ± 6.21), which yielded a lower survival rate.

In contrast, the poring pulses with the greatest values for construct integration were the 40 V and 35 V (11.24 % ± 2.76 and 12.81 % ± 2.80 respectively), which were statistically equivalent but substantially different from the poring pulse that yielded the lowest integration value, the 30 V (0.74 % ± 0.54).

When all the data from these two plots were combined, the only common value was the 35 V poring pulse, which was the voltage that allowed the best integration rate to be obtained without jeopardizing the embryo's viability.

3.2.9 Comparison to microinjection

After the protocol had been improved and had already shown encouraging results in terms of survival and construct integration, it was time to compare the procedure to the most frequently used delivery method, the microinjection. For this, 1-cell stage zebrafish embryos were either injected (n=8 rounds of 30 embryos each) with 10xUAS:LSSmOrange at 18 ng/μL, or electroporated with the same construct at 300 ng/μL in PBS applying 35 V as poring pulse and 5 V as transfer pulse at the same cell stage (n=8 rounds of 30 embryos each). The rates of survival and integration of both methods were assessed and compared, and the findings are shown in Figure 3.18.

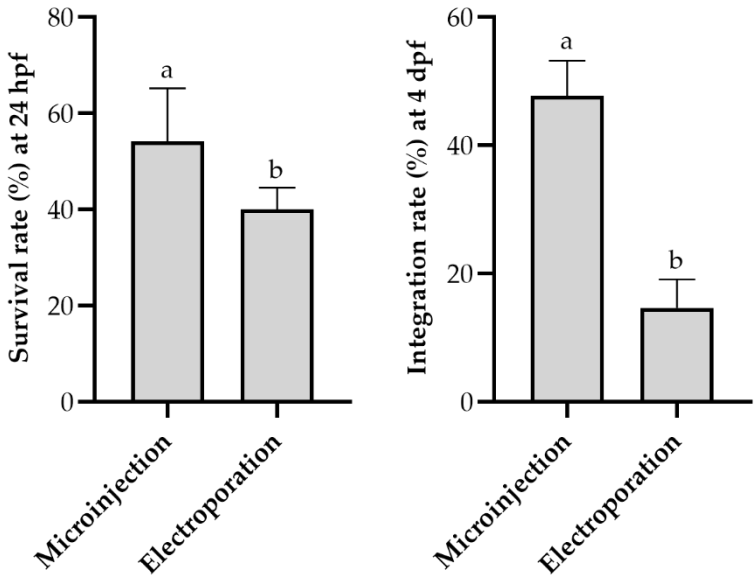

Figure 3.18- Left: Comparison of the survival rates (mean ± standard deviation error) at 24 hpf, between microinjection and electroporation of 1-cell stage zebrafish embryos; Right: Comparison of the transient integration rates (mean ± standard deviation error) of 10xUAS:LSSmOrange at 4 dpf, between microinjection and electroporation of 1-cell stage zebrafish embryos. Different letters (a and b) indicate statistically significant differences compared to all other groups. All conditions were tested eight times (n=8) each time using 30 embryos. An unpaired t-test was performed, both left and right, with significance set at p-value < 0.05.

When comparing the survival rates of each method, microinjection (54.19 % ± 10.96) exceeded electroporation values (40.00 % ± 4.52) by a significant margin. This was also true regarding the integration rate, where microinjection (47.72 % ± 5.47) significantly outperformed electroporation (14.60 % ± 4.46).

Despite this, the use of electroporation as a plasmid DNA delivery method produced quite promising results, suggesting that with further optimizations to the protocol, it could be a suitable alternative to microinjection. The outcomes of Tg(isl2b:Gal4;10xUAS:LSSmOrange) generation in one-cell stage embryos using *in vivo* electroporation and microinjection as delivery methods are shown in Figure 3.19.

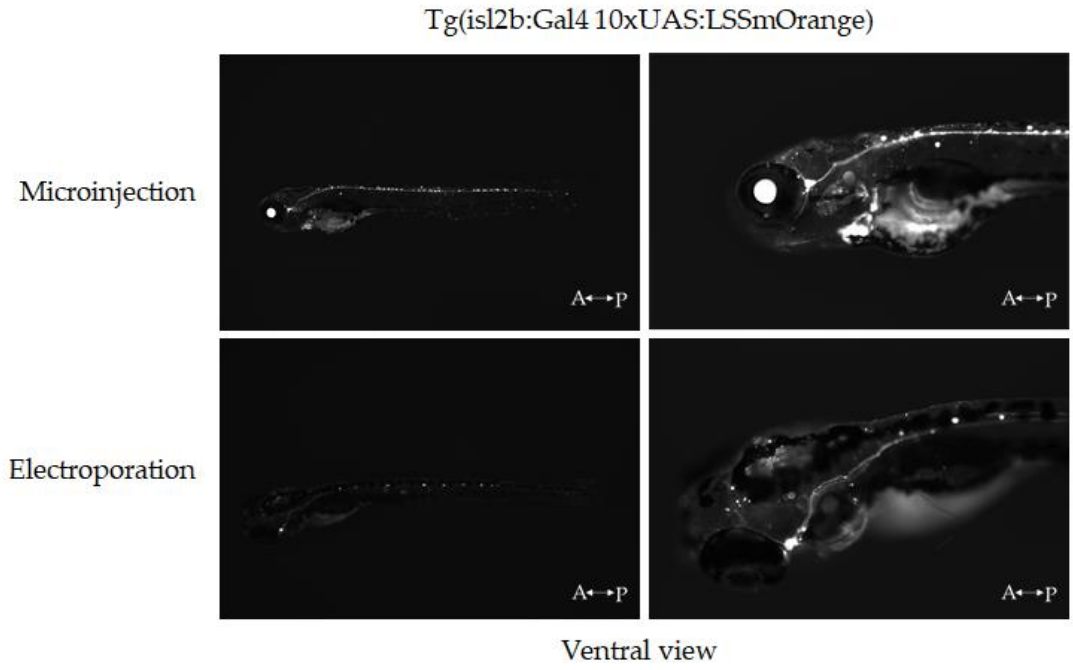

Figure 3.19- Expression pattern of the Tg(isl2b:Gal4;10xUAS:LSSmOrange) generated by electroporation and microinjection into one-cell stage embryos. Transgenic zebrafish larvae with a close to complete expression pattern screened at 6 days old. A: anterior; P: posterior.

When comparing the screen outcomes of electroporation with microinjection, the first thing that stands out is that there was no significant difference in terms of fluorescence intensity or expression pattern. Expression was seen in the retina, as well as the spinal cord and trigeminal nerve, in the microinjection images, whereas in the electroporation images, expression was limited to the spinal cord, which is not as extensive as the microinjection images and has a lower fluorescence intensity, and expression on the trigeminal nerve, similar to the phenomenon seen in microinjection.

This indicates that, despite having a lower integration rate, electroporation can yield expression patterns that are comparable to microinjection. It's worth noting, however, that the acquired images in both cases show the most complete expression pattern as well as the maximum fluorescence intensity detected in the clutches during the screening. This means that the images shown here do not reflect the entire population of screened larvae, but rather the most successful ones.

When the results from the screens and the statistical data are combined, it becomes clear why the microinjection technique obtained better results, even though there were no larvae with a complete expression pattern. The microinjection technique produced mostly larvae with a high fluorescence intensity and a close-to-complete expression pattern. In the instance of electroporation, the vast majority of larvae displayed a very incomplete pattern of

expression, restricted to the trigeminal nerve accompanied by a slightly weaker fluorescence signal.

4

DISCUSSION

4.1 Generation of a new neuronal transgenic zebrafish line

The use of GCaMPs allows for the detection of oscillations in calcium concentration, indicative of neuronal activity. During the course of several years, newly improved versions of GCaMPs have emerged [65], [67], [68]. Fast-responding indicators, such as GCaMP6f, provide more accurate tracking of fluctuations in firing frequencies in neurons with higher levels of activity. In this work a new neuronal transgenic zebrafish line was generated by microinjection into 1-cell stage embryos. The elavl3:GCaMp6f EF05 construct comprises an improved version of the original fast GCaMP6, called GCaMP6f EF05, which has a greater signal-to-noise ratio and dynamic range. This calcium indicator is under the control of a promotor with a pan-neuronal expression pattern, elavl3 (or previously called HuC). The GCaMP6f EF05 was created by using site-directed mutagenesis to introduce mutations in the EF05 loop domain of the GCaMP6f sequence (Tomás *et al.*, unpublished data) and the alterations in the EF05 loop domain were previously documented in GCaMP3 [65].

4.1.1 Microinjection into one-cell stage embryos (F-1 generation)

In order to generate a stable transgenic zebrafish line embryos, obtained from a wild-type cross, were injected with the elavl3:GCaMP6f EF05 construct into one-cell stage embryos in the presence of Tol2 transposase mRNA to ensure construct integration into the genome.

According to Kawakami and colleagues (2004), transgenesis using the Tol2-mediated transposon system exhibits the highest germline transmission frequency and suffers fewer silencing effects across generations, compared to what is obtained by other transgenesis systems [115]. It was even demonstrated that when co-injected with transposase mRNA and

plasmid DNA with Tol2 arms, over 50% of injected zebrafish transmitted the transgene insertions to the offspring.

The optimal concentration at which the DNA was injected had already been established by a colleague in the lab, through several rounds of injections, adjusting the concentration until positive results were obtained. Even after that optimization, this was a particularly challenging construct to integrate due to its large size. Given the observed high mortality and malformation rates in injected embryos, it might mean that the optimal established concentration (50 ng/µL) is a toxic concentration for embryos. However, lower concentrations were tested previously in the lab but were unsuccessful. This would mean that even if the construct was integrated with high efficiency, it would not be possible to detect on the screens due to the fact that most embryos carrying the construct would not resist the toxicity of the concentration at which it was injected. This is supported by the data in Table 3.1, in which it is possible to see that, despite the high number of injected embryos in each round of injections, hardly a few have managed to survive until the moment of the screen. The highest survival rate obtained from 8 (data available) injection rounds comprising more than 3500 injected embryos is only of 24.1 %. Hence, emphasising the importance of the few lines we were able to generate from injections.

4.1.2 Selection of fish for line establishment

Injected embryos will harbour an uncertain number of independent insertions, thus, to accomplish predictable inheritance of functional transgenes involves several generations of outcrosses and each fish of the first generation is considered a unique transgene carrier. Single insertions tend to me more stable than multiple ones. Generally, microinjected zebrafish (F-1 generation) are screened for transgene transmission by being outcrossed to wild-type zebrafish. At 72- to 96- hpf, the progeny is screened and the larvae exhibiting the strongest fluorescence intensity along with the complete expression pattern are raised into adulthood (F0). When zebrafish from the F0 lines have achieved sexual majority they are crossed to wild-type strains to evaluate in the progeny if germline transmission had occurred. F0 parents with an intense and complete transgene transmission to the next generation (F1) are called founders (F0). Individual F0 animals that provide offspring are consecutively numbered and kept in isolation. Their F1 embryos are afterwards screened for transgene integration by fluorescence microscopy. If a reasonable clutch size (~100 embryos) is transgene-negative, the corresponding F0 animal is not considered. Whereas if the clutch is positive, F1 embryos are raised, and the F0 parent is kept for further rounds of outcrosses. This cycle continues until the

line is considered stable which is when transgenic fish comprise a single insertion. Single-insertion zebrafish transgenics feature predictable inheritance patterns and expression, yet long-term transgene stability is critical to sustainable experimentation [35]. Single insertions can be confirmed by Southern blot analysis [28]. Transgene silencing happens randomly and affects the value of any established line. Hence, establishing at least three independent transgenic lines for any given transgene is recommended.

In establishing transgenic lines, each generation is crossed with wild-type strains, giving rise to new generations of heterozygous transgenics. These outcrosses can cause a decrease in the intensity of displayed fluorescence, if care is not taken in its selection; for this reason the larvae are selectively chosen to be reared until adulthood. In the injected larvae, the strongest, more complete and uniform the expression is, the more likely the construct is to have integrated all cells, including the germline cells, and hence to give rise to positive offspring. However, it is important to note that the fact that the construct is integrated into the genome does not mean that it is expressed. Depending on the region where it was integrated, which is random in the case of transgenesis mediated by tol2 transposon system, it may suffer silencing. On the contrary, for selecting injected adults, among those with germline transmission, to continue the line establishment, the criteria are not only a complete expression pattern and a good fluorescence intensity, but also the percentage of positive progeny, that should be as low as possible. This is because, normally, a lower percentage of positives is indicative of integration in a single location, which generates a more stable transgenic population. In case there is integration in more than one site, the probability of this fish originating positive offspring is greater, and each time this fish is crossed, its offspring will present different expression patterns generating a heterogeneous clutch, which is a factor of instability in establishing a line. The goal while establishing a line is to generate stable transgenics in their expression pattern and fluorescence intensity so that the line can be characterized. The F-1 generation is still very unstable in the sense that there are, if it has integrated, cells of the germinative line with integration in different locations, which generates gametes with integrations in different locations and consequently generates individuals with different expression patterns (mosaicism at the level of the pattern of expression). Through the screens and rigorous selection of progeny raised into adulthood that follow the establishment of the line, it is possible to stabilize the line over the generations.

4.1.2.1 A promising F-1 line

During the first steps in establishing the line, there was an F-1 line (5951 line) that stood out for displaying a high level of fluorescence intensity and presenting a complete and homogeneous expression pattern at the pan-neuronal level. Interestingly, it also presented a relatively high percentage of integration, 16 %, considering it was a line at the beginning of establishment. Contrary to this line, all the others generally presented a variable expression pattern with a low fluorescence intensity, accompanied by a low percentage of positive offspring. These data alone were already an indication that something was not right; however, that was only realized later. That being said, not only was this line continued to be established, but it became the line of preference and on which the establishment of the transgenic line elavl3:GCaMP6f EF05 was focused. Of the various F0 lines generated from line 5951, there were some F0 fish that proved to be particularly promising in terms of the percentage of positive offspring generated, which, similarly to the line that precedes it, was relatively high, 31.7%. Naturally, with the establishment of lines, generations get increasingly stable and therefore the number of positive offspring they generate is increasingly higher. Having that into account and considering the already high value obtained in F-1, this value was not strange, and larvae were allowed to grow to adulthood. When the transgenic fish of this F1 generation, originating from line 2461, finally reached sexual maturity, they were crossed with a wild-type for the final confirmation that the line was finally established. Larvae were pre-screened and handed over to an investigator who analysed them using light sheet microscopy. Surprisingly, the results showed we were in the presence of a line with nuclear-localized pan-neuronal expression. Knowing that the injected elavl3:GCaMP6f EF05 construct had no nuclear tag in the sequence, the only way to have a nuclear expression detected in the establishment of the Tg line (elavl3:GCaMP6f EF05) would be if we were in presence of a contamination. Interestingly, around this time, a line was discovered in the facility that had been genetically contaminated (5252 line). We confirmed that was the line used for the injections from which positive larvae gave rise to the line 5951, the precursor line F-1 of all the line establishment done so far. Sequencing results further confirmed such findings. The sequencing results revealed that the integrated construct in the transgenic line was elavl3:H2BGCAMP, confirming the results obtained by light sheet microscopy. Histone 2B sequence (H2B) is a commonly used tag to direct the fluorescent reporter expression to the nucleus [53], [55].

Considering the sequencing results and analysing the lines already established in the facility that could serve as a source of genetic contamination, it was concluded that the line was contaminated with elavl3:H2BGCaMP6s or elavl3:H2BGCaMP6f. Despite the detected

construct showing a similar expression pattern and emitting fluorescence in the same region of the spectrum, by analysing more closely the screens that were made along the establishment of this line, the lack of expression in the optic tectum region was visible, which could be translated in two situations; we were either in the presence of a silenced line, or we were in the presence of a nuclear-marked line. The exact source of genetic contamination, which of the GCaMP, could be found for example, by means of a PCR with specific primers for one of the GCaMPs, 6f or 6s. However, for the purpose of this work to know what the exact genetic contamination was not essential and was therefore a waste of resources; for this reason such confirmations were not made. The important next step was to go back to the beginning and choose a new F-1 line, which did not show signs of genetic contamination, and which did not have the line 5252 as a parent, to start a new line establishment.

4.1.2.2 Finding a new F-1 line to establish

The most promising F-1 line was the 6032 line which exhibited a complete cytoplasmic-localized pan-neuronal expression pattern accompanied by a moderate green fluorescence intensity, which is typical of a line in the early stages of formation. In addition, this F-1 line produced a low percentage of integration in the progeny, 4.2 %, which could translate into stronger integration stability. The sole fish from the 6032 line was outcrossed with a wild-type strain so that the germline transmission could be evaluated in the progeny. In contrast to the previously established line, this new F-1 line exhibited a diffuse and homogenous expression pattern observed in all nerve cells, which indicated that the integrated construct did not have a nuclear marker in its sequence. When the transgenic fish from the raised F0 line (2336 line) reached sexual maturity, the founders were outcrossed with a wild-type strain to evaluate whether there was germline transmission. All 2336 offspring displayed a complete pan-neuronal expression pattern associated with homogenous and high fluorescence intensity. In order to confirm these findings, and given the line establishment history, a sample of the caudal fin from a 2336 founder was collected and sequenced. The results supported screen findings, revealing that the integrated construct in this new line was the elavl3:GCaMP6f EF05 construct. For that, the line establishment proceeded: once reached sexual maturity, the most promising F1 line (2830 line) was outcrossed and the 4 days old offspring larvae were pre-screened for pan-neuronal expression and, given the previously contamination scenario, taken to the confocal microscope for image acquisition. The larvae from the F1 progeny of the newly established Tg(elavl3:GCaMP6f EF05) 2830 line displayed the characteristic elavl3 pan-neuronal expression pattern exhibiting a cytoplasmic-localized fluorescence in all nerve cells. In this line,

contrary to what was observed in the first line establishment attempt, the optic tectum (TeO) area exhibited fluorescence. The optic tectum region is rich in neuron projections and with low content of neuron nucleus [116]–[118]. As a result, in the presence of a nuclear marker, more specifically a calcium indicator, this region does not express the marker, resulting in the absence of fluorescence. Hence, fluorescence in this area indicates that the expression had in fact occurred at a cytoplasmic level, which meant we had successfully established the elavl3:GCaMP6f EF05 line throughout the course of 3 generations. These results were corroborated by sequencing results that confirmed the correct transgenic line establishment.

4.2 Optimizations to the electroporation protocol in zebrafish embryos

The first successful employment of electroporation to generate transgenic animals was accomplished in 1990 by Inoue and colleagues in medaka fish [119]. Ever since, electroporation has been successfully used in other animal models including xenopus, chicken and even mammals, by electroporating localized areas, *in musculus* electroporation for example, or entire organisms, *in ovo* electroporation [120], [121]. In zebrafish, electroporation has often been used to incorporate loss of function reagents, to edit gene expression, and to deliver dyes and expression plasmids to large numbers of cells in different regions of the developing larvae and adult fish [122]. Nevertheless, gene transfer into zebrafish embryos by electroporation has rarely been reported previously [112]. Last year, shortly before the beginning of this project, Zhang, C. and colleagues published their work on "Transgenic Expression and Genome Editing by Electroporation of Zebrafish Embryos". Although being an important advance in the development of a new mechanism for efficiently delivering nucleic acids into fish embryos, this protocol could be improved to be more advantageous to the scientific community as an alternative to microinjection. Features such as the number of embryos electroporated simultaneously (around 15 embryos) and the advanced embryo cell stage used (8 to 16-cell stage) should be improved. In this dissertation, these conditions were optimized to overcome the limitations of the previously published protocol.

4.2.1 Dechorionation method

A disadvantage of the electroporation delivery method in zebrafish embryos is the fact that, in opposition to other animal model's embryos, zebrafish embryos comprise two

membranes: the cell membrane (inner membrane) and the chorion (external membrane). These two membranes constitute two physical barriers that genetic or molecular materials need to overcome in order to arrive to the final destination which are embryo cells. For this reason, it has been reported for over 40 years the necessity to weaken and remove zebrafish embryo's chorion [119]. The most used method for dechorionation, also reported in Zhang, C. *et al.,* 2020 work, is an enzymatic method. This method consists of incubating embryos with an enzymatic mixture called pronase that comprises proteases and peptidases capable of hydrolysing most peptide bonds [123]. Depending on the time of incubation, the chorion may not be weakened enough to be able to be removed, if a short period of incubation is applied, or it can lead to the digestion of the embryo itself, when left to incubate for a long period. The optimal time needs to be long enough to digest the chorion but not too long to prevent embryo´s digestion [112]. However, not all embryos are the same and some might be more sensitive to pronase than others, resulting in slightly different time points of chorion digestion. To prevent embryos from dying due to damage of the yolk sac, we tested dechorionation through a different method where embryos had the chorion removed through carefully manual piercing and tearing, without interfering with the embryo, using sharp forceps. The two methods were then compared, in terms of embryo survival, and the results revealed that both methods involve some level of embryo's mortality (Figure 3.7); however, the mortality rate was significantly superior when the manual dechorionation method was employed. These results are in concordance with the ones reported by Zhang and colleagues [112]. In fact, the dechorionation method using pronase incubation is much easier to control and reproduce, by adjusting the concentration of pronase used and the incubation time, than the manual dechorionation method, which is entirely dependent on the operator's skills. From this moment on, the dechorionation method performed in all the following optimizations was the incubation with pronase which contributes to an increase of electroporation's protocol reproducibility. It is worth noting that when comparing the two methods of dechorionation, only survival was considered; because comparing the effectiveness of the two techniques would be inaccurate. The operator is fully responsible for the success of manual dechorionation, which means that as many embryos as the operator desires are dechorionated. This circumstance may even be advantageous if it were not for the fact that the enzymatic approach is faster and more practical to perform; by changing the volume of pronase used, it is feasible to remove the chorion of as many embryos as desired in just 15 minutes, which would take hours to do manually.

Following Zhang, C. *et al.*, 2020 protocol, embryos dechorionation was being performed through pronase incubation under the agitation of a shaker [112]. However, agitation

represents a mechanical process that could impact embryos stability and ultimately survival. To better understand this effect, 8-cell stage zebrafish embryos were incubated in pronase with and without agitation. The results (Figure 3.8) showed that the method performs equally well with or without agitation. As a result, we chose not to agitate the embryos throughout their dechorionation in our study.

4.2.2 Number of embryos electroporated at once & DNA solution volume required

In this work, the electroporation chamber used (Figure 2.4 C) differs from the chamber used by Zhang in terms of dimensions, more specifically, the length [112]. For this reason, both the number of embryos that would be electroporated in each electroporation cycle, as well as the volume of the DNA solution had to be established. The number of embryos used in each electroporation cycle, with a few exceptions, was 30 embryos. This value was chosen because it allows the entire length of the chamber to be occupied without the embryos touching the walls of the chamber, which would compromise their viability [89]. The volume of DNA solution in the electroporation chamber was set at 150 µL and it represents a compromise between using the minimum necessary volume of DNA and ensuring that the entire surface of the embryos as well as the entire perimeter of the chamber would be covered with solution. This is particularly important because, (1) as the chorion was previously removed, embryos are more likely to have their viability compromised; and (2) if the DNA solution does not occupy the entire perimeter of the electroporation chamber, the transmission of the applied electrical pulse is compromised.

In addition, the volume of solution used in the electroporation chamber constitutes an important indirect way of controlling the difference between the applied electrical pulse and the voltage felt by the embryos. The dilution medium used for electroporation is, as a rule, a saline medium, rich in ions, and is therefore a medium that conducts electrical current. For a given concentration of solution, the increase in volume will allow the ions to be more dispersed, thus facilitating the passage of electric current, which reduces the resistance of the system, which makes the voltage felt by the embryos similar, in intensity, to the applied voltage [96]. This way, the volume of DNA solution indirectly controls the impedance values of the system, which is the resistance felt to the passage of current, affecting the efficiency of the technique.

4.2.3 Embryo cell stage

The use of embryos during cleavage stage is not ideal for the generation of stable transgenic lines [112]. To increase the likelihood of integration into the germline, which is needed to produce stable transgenic lines, delivery must be done in the stage of one cell [124], [125]. If so, every cell in the organism will have the transgene since all will originate from that first cell. For that, electroporation procedural steps were carried out using both 8- and 1-cell stage under the same conditions for comparison purposes. No integration was observed for neither of the cell stage embryos used; however, as demonstrated in the Figure 3.10 the use of embryos at 1-cell stage had a positive effect on embryo survival in all the steps during and after electroporation itself. This is clearer in Figure 3.11 where the overall survival rate of the electroporation delivery method was assessed for both 8- and 1-cell stages. The experiment using 1-cell stage embryos yielded a significantly higher survival rate, almost 2 times higher, than when 8-cell stage embryos were used. For this reason, the use of one-cell state embryos was adopted for the following optimizations made to this protocol.

However, it is important to note that when compared to the control group, both experimental groups demonstrate a significantly low survival rate, which is indicative of the need for further protocol optimizations. One of the variables that may have intervened in the embryo survival rate was the fact that both the petri dishes from where the embryos are transferred to and from the electroporation chamber and the micropipette tips used in transporting and handling the embryos are made of plastic. Embryos are not as mobile without the chorion and become stickier, and since the plastic is not an inert material, it will promote embryo's adhesion which, when wrecked by the movement of the embryos in the various stages of electroporation, will cause embryo's inner membrane rupture and compromise its viability. One way to avoid this situation would be the exclusive use of glass materials, which would make the process more expensive.

4.2.4 Electrical pulses

The electroporation-mediated gene delivery depends highly on electrical parameters including pulse voltage [92], [97], [102]. NEPA21 electroporator allows two-step pulse electroporation: high-voltage pulses of short duration (poring pulses) that creates micro holes on the cell membrane; and low voltage pulses of long duration (transfer pulses) that promotes the delivery of nucleic acids into the cell. Throughout the transfer pulse, the change of polarity orientation increases the chance of transfection [126]. Furthermore, the device enables the

measurement of the impedance between the electrodes, which should always be kept under 0.2 kΩ [112].

Higher voltages lead to a decrease in survival, while lower voltages lead to the inefficiency of the process [112]. For that, it was necessary to find the optimal set of electrical pulses. To accomplish so, the transfer pulse was set at 10 V, while the poring pulse was gradually increasing. The rest of the electrical parameters were kept constant throughout all optimizations, and equal to the ones reported by Zhang and colleagues. Results showed (Figure 3.12) that when the transfer pulse was held at 10 V, a general poor survival rate was yielded being in accordance with literature [112]. Hence, the two poring pulses that retrieved higher survival rates when using 10 V of transfer pulse were also tested but using a 5 V transfer pulse instead. No integration was observed in either case; however, the survival rates were assessed and compared (Figure 3.13). For the same set of poring pulses, when the lowest transfer pulse was applied, the best survival rates were yielded. These results are supported by the literature reporting that survival rate declines significantly when the voltage of transfer pulse increased from 5 to 10 V [112], [127]. These experiments settled the transfer pulse voltage for the optimizations to come, set at 5 V. However, the poring pulses used so far were retrieving poor survival rates, and for that reason, a new experiment was performed to test other potential optimal poring pulses. According to the literature lower pulses produced minimal lethality and bellow 30 V there is a sharp decrease on the transfection rate, which is, in part, supported by our experimental results [128]. Once more, no integration was observed for neither of the poring voltages tested; however, the survival rates were assessed and compared (Figure 3.14). No significant differences on survival rates were found when 25, 30 and 35 V pulses were applied. These 3 poring pulse voltages retrieved the highest survival rates, followed by the 40 and 50 V poring pulses showing no significant difference among them too. Finally, the poring pulse voltage value that proved to be unsustainable for embryos survival, the 75 V pulse retrieved the lowest survival rate. To maximize the probability of integration, risking it could be toxic, and since no difference was found in the survival rates between the 40 and 50 V, the chosen pulses set for the following optimizations were 50 V of poring pulse followed by 5 V of transfer pulse.

4.2.5 Dilution medium

According to several authors, the composition of the electroporation medium is one of the most critical parameters for electroporation-mediated transfection efficiency [92], [102].

The medium's pH is also important and should be kept at a physiological level, usually around 7.2 [102]. Until this point, a DNA concentration of 180 ng/µL was being used given that it was the working stock concentration already available in the laboratory and was close enough to the plasmid concentration used by Zhang, C., *et al*. 2020. Nevertheless, the working stock ran out and for that reason a new working stock had to be prepared from a concentrated stock. For this, the three media available in the research facility were tested as dilution medium for the DNA solution throughout impedance measurement (Figure 3.15). Out of all the tested solutions, the DNA solution diluted in PBS was the one with the lowest impedance value, which translates into lower resistance to current flow and, consequently, greater transfection efficiency. This is because, the greater the salt composition, the greater the ion content, which makes the medium a good conductor of electrical current [96]. Good conductors decrease the resistance to the passage of current called impedance, thus allowing the embryos not only to feel a more uniform field, but also bringing the voltage felt by the embryos closer to the actual voltage applied by the electric pulse. For this, PBS became the DNA dilution medium of choice in the remaining optimizations to the electroporation protocol.

4.2.6 DNA concentration

According to the literature the electroporation-mediated transfection is dose-dependent, meaning that the efficiency of the method is affected by DNA concentration [112]. For this reason and taking advantage of the fact that new DNA working solutions had to be made, different DNA concentrations were tested starting at the concentration used until then and increasing, for comparative purposes. The electroporation survival and integration rate were determined and compared (Figure 3.16). The results show that for a DNA concentration bellow 350 ng/µL, the survival rate is as higher as the integration rate is lower. The lowest DNA concentrations are the least efficient, whereas the highest concentrations are the most lethal, being toxic to the cells, so a balance had to be found. Taking this into account and by evaluating the graphs (Figure 3.16) as well as the statistical values, the DNA concentration that allows to retrieve the best integration rate without compromising the survival rate is 300 ng/µL. It presents the highest rate of integration, together with 350 ng/µL, and the second highest survival rate. This was the new concentration adopted in the optimizations that followed.

4.2.7 New poring pulse

The application of electrical current in living beings is never beneficial and therefore it is always desirable to reduce its impact, lowering the current as much as possible without compromising the results. It was essential to re-optimize the electrical pulse now that the DNA solution medium and concentration had been optimized in a way that integration was observed for the first time. Three poring pulses were tested by employing a transfer pulse of 5 V, previously defined as optimum voltage for the transfer pulse. The electroporation integration efficiency and embryo survival resulting from the application of three different poring pulses were determined and compared (Figure 3.17). The poring pulse that allowed the best integration rate to be obtained without jeopardizing the embryo's viability was the 35 V poring pulse.

4.2.8 Comparison to microinjection

After all the optimizations to the electroporation protocol that led to encouraging results in terms of survival and construct integration, the electroporation delivery method was compared to the most frequently used method, the microinjection. The rates of survival and integration of both methods were assessed and compared (Figure 3.18). Results showed that the electroporation technique was not as powerful as microinjection when it came to embryo survival and integration efficiency. Nevertheless, screen results showed that electroporation obtained similar fluorescence intensity even though exhibited an incomplete expression pattern compared to microinjection results (Figure 3.19). Microinjection is a classical delivery technique; however, it requires the researchers to have specific manual skills to prevent cell damage as well as being time consuming for large-scale applications, as embryos need to me injected one at a time [126], [129]. Whereas electroporation does not rely so much on the operator skills, it is simpler and easier to employ, and can be beneficial when the embryos viability is a priority, since almost every parameter can be controlled and improved, even though requiring chorion to be removed from embryos prior to electroporation procedure and uses large volumes [124], [126], [129], [130]. For all the mentioned advantages, it is important to not cease the efforts for optimizing the electroporation protocol, in hopes it might someday be as efficient as the microinjection delivery method.

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