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Establishment of Neural Circuits During Zebrafish Development: Characterization and Optimization of Imaging Conditions

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Resumo

O peixe-zebra (*Danio rerio*) é um peixe de água doce da família dos ciprinídeos. Em neurociência, o uso do peixe-zebra como modelo tem vindo a aumentar, pois este apresenta um padrão anatómico e circuitos neuronais semelhantes à maioria dos vertebrados. Para além disso, a sua transparência natural em estados lavares permite a imagiologia do cérebro *in vivo*. Devido à sua corrente popularidade, existem várias ferramentas genéticas disponíveis que permitem a criação de linhas transgénicas estáveis.

No peixe-zebra, o desenvolvimento do cérebro ocorre num curto período de tempo. A neurogénese inicia-se por volta das 10 horas após fertilização (hpf), ainda durante a gastrulação. Nesta etapa forma-se um modelo primário da rede neuronal. A axogénese destes neurónios inicia-se entre as 14 e 24 hpf. A neurogénese secundária, expande a rede neuronal primária, não existindo separação temporal clara entre as duas fases.

Durante o 1º dpf o tubo neural é formado e sofre morfogénese regional criando 10 neurómeros entre o prosencéfalo, mesencéfalo e rombencéfalo. Esta regionalização (rostro-caudal e dorso-ventral) contribui para o desenvolvimento dos vários subtipos de neurónios e células gliais uma vez que fornece gradientes de fatores parácrinos e sinais celulares. Assim, no final do 1º dpf, os embriões já apresentam contrações musculares espontâneas, ao 2º dia de desenvolvimento os embriões respondem ao toque e no final da primeira semana de desenvolvimento, as larvas exibem um conjunto de comportamentos inatos, entre os quais os comportamentos visualmente-guiados.

Os comportamentos visualmente-guiados são comportamentos exibidos naturalmente pelas larvas do peixe-zebra e podem ser controlados em laboratório. A resposta optocinética é um movimento reflexivo do olho que permite que o organismo siga um estímulo rotativo. A resposta optomotora é uma adaptação do comportamento natatório a uma mudança percecionada no movimento da água.

Para compreender como o cérebro controla estes comportamentos é importante identificar os circuitos que estão por detrás destes e isto é possível com a criação de linhas transgénicas. As linhas transgénicas aqui referidas foram geradas pelo Orger Lab (Fundação Champalimaud – Centre for the Unknown) através da introdução por transgénese mediada por transposão de um fragmento genético de peixe-zebra.

O fragmento genético introduzido contém um gene de interesse, que se sabe previamente que tem expressão em certas populações neuronais. Associado ao primeiro codão deste gene de interesse está o GFF (derivado de Gal4). Por cruzamento destas linhas transgénicas com uma linha UAS:GFP, é possível exprimir o repórter (GFP – *Green Fluorescent Protein*) nas populações neuronais onde o gene de interesse é naturalmente expresso.

Após imagiologia, é necessário atribuir a cada estrutura uma região cerebral num processo chamado de caracterização anatómica. Neste momento, vários atlas digitais e bases de dados do cérebro do peixe-zebra têm sido desenvolvidos para o estádio de 6 dpf, como o Z-Brain, Zebrafish Brain Browser e o Mas-Planck Zebrafish Brain Atlas. No entanto, para estádios mais precoces de desenvolvimento, apenas tentativas parciais foram realizadas.

Desta forma, os objetivos desta dissertação são: caracterizar anatomicamente duas linhas transgénicas (Tg(Pitx2c:GFF) e TgBAC(sst1:GFF) geradas pelo Orger Lab) e explorar marcações de contraste e

posições de montagem adequadas para o estudo dos estágios de desenvolvimento precoces em peixe-zebra.

As imagens da caracterização anatómica foram adquiridas através de microscopia confocal após um tratamento imunohistoquímico e de seguida submetidas a análise de imagem e caracterização anatómica. Por comparação visual com bibliografia, atlas e bases de dados previamente descritos, a caracterização anatómica foi efetuada, identificando estruturas cerebrais no prosencéfalo, mesencéfalo e rombencéfalo.

Na caracterização anatómica, ambas as linhas mostraram expressão de GFP desde o 1º dpf até ao 6º dpf. Nos primeiros dias de desenvolvimento a expressão é, no geral, mais fraca, sendo que a sua distribuição espacial e nível de expressão aumentam com o passar do tempo. O padrão de expressão de GFP parece ser estabelecido em torno do 3º e 4º dpf. Após estes estágios, o padrão espacial mantém-se verificando-se apenas o crescimento em número de células até aos 6 dpf.

O gene Pitx2c codifica um fator de transcrição. A linha Tg(Pitx2c:GFF) tem expressão de GFP em regiões cujo gene – Pitx2c – já tinha mostrado expressão prévia. Entre as quais, destaca-se a assimetria encontrada no diencéfalo (mais propriamente no epitálamo), a expressão no núcleo do fascículo longitudinal medial (nMLF), no núcleo oculomotor e na retina. Todas estas estruturas anatómicas estão envolvidas nos circuitos que formam os comportamentos de resposta optocinética e optomotora.

O gene sst1 codifica o neurotransmissor de somatostatina. A linha TgBAC(sst1:GFF) também apresenta expressão em estruturas previamente descritas com expressão do gene, como o cerebelo. Outras regiões, como epitálamo, pálio, sub-pálio e núcleo interpeduncular também foram aqui verificadas. Estas estruturas, encontram-se associadas às redes neuronais que levam aos comportamentos visualmente-guiados.

Devido ao facto dos atlas e bases de dados do cérebro do peixe-zebra se focarem sobretudo em estádios de desenvolvimento mais avançados, não existe ainda um protocolo otimizado para a imagiologia destas amostras quer em termos de marcação de contraste, quer em termos de montagem entre lâmina e lamela. Aqui, tentou-se explorar algumas alternativas a estes casos.

O tERK é um anticorpo usado por vários atlas digitais como marcação de contraste. Contudo, e apesar de ser uma boa marcação para estádios mais avançados de desenvolvimento (como os 6 dpf), o seu desempenho não é tão favorável para estádios mais precoces (como os 2 dpf). Deste modo, alternativas que possam ser usadas para todos os estádios de desenvolvimento são necessárias. Aqui explorámos vários tipos de marcadores de contraste: mScarlet (uma proteína fluorescente associada ao promotor da α-tubulina, na linha transgénica: alphatubulin:mScarlet), DiD (um corante lipídico que marca membranas celulares) e BODIPY TR *methyl ester* (um corante que marca membranas de organelos celulares).

Em relação às marcações de contraste alternativas à marcação por tERK (anticorpo), alphatubulin:mScarlet parece ser uma potencial alternativa, uma vez que permite a marcação desde estágios precoces de desenvolvimento até estágios mais avançados e também permite não só a imagiologia de tecido fixo como também permite a imagiologia *in vivo.* DiD, também apresenta algumas vantagens em relação ao tERK, podendo ser tido como alternativa a este. O BODIPY TR, pelo contrário, não parece ser uma alternativa adequada.

Em termos de posicionamento durante a montagem dos embriões para imagiologia, experimentaram-se duas orientações diferentes: horizontal (convencional) e vertical (alternativa). Isto, porque o cérebro dos embriões de peixe-zebra apresenta uma curvatura que faz com que haja estruturas que fiquem menos

acessíveis à imagiologia. Ambas as posições permitiram a imagiologia de grande parte do cérebro, podendo ser alternadas dependendo das estruturas que se pretende estudar.

Ainda em relação à imagiologia dos cérebros de estádios precoces de desenvolvimento, usou-se a microscopia confocal e de *light sheet*. A microscopia confocal apresenta uma resolução elevada que permite o estudo detalhado das estruturas cerebrais, enquanto que a microscopia de *light sheet* permite a rotação da amostra dentro do microscópio permitindo a imagiologia a partir de vários ângulos, para uma mesma amostra.

Em suma, através da caracterização anatómica e da exploração de alternativas para estádios de desenvolvimento precoces, tencionou-se contribuir em parte para o enriquecimento das bases de dados já existentes com a caracterização de duas novas linhas transgénicas e em parte para a melhoria da imagiologia do cérebro precoce dos embriões de peixe-zebra. Isto, tendo em conta o objetivo geral do laboratório (Orger Lab) de construir atlas anatómicos digitais para os vários estádios de desenvolvimento e para cada linha transgénica.

Palavras-Chave: Desenvolvimento; Circuitos Neuronais; Caracterização Anatómica; Peixe-zebra

Abstract

The use of the zebrafish (*Danio rerio*) as a model in neuroscience has been growing since it presents a similar anatomic pattern and neuronal circuitries to most vertebrates. In zebrafish, development occurs in a short period of time: neurogenesis starts during gastrulation. At the end of the 1st day post fertilization (dpf), embryos already exhibit spontaneous muscular contractions and within one week of development, larvae display a set of innate behaviors, such as the visually guided behaviors. The visually guided behaviors are naturally performed and easily controlled in the laboratory, making these easier to study and understand the neuronal circuits behind them.

Additionally, the expansion of the genetic tools available has been favorable to the construction of new transgenic lines that allows the identification and imaging of neuronal populations of these networks. After identifying these populations, it is necessary to assign brain regions to each structure with the aim to build a comprehensive atlas through development. Several atlases and databases of the zebrafish brain have been created for the 6 dpf stage, but for early stages only partially attempts have been done.

Here we address the anatomical characterization of two GFP-expressing reporter lines – Tg(Pitx2c:GFF) and the TgBAC(sst1:GFF) – with the long-term goal of creating a 3D digital atlas from the 1st dpf to the 6th dpf. These images were acquired by confocal microscopy after immunohistochemistry. Then they were submitted to image analysis. Through visual comparison with previously described bibliography and atlases, anatomical characterization of our images was done, identifying brain structures in the prosencephalon, mesencephalon and rhombencephalon.

Furthermore, we explored counter staining alternatives to tERK antibody – contrast staining – such as the mScarlet, DiD and BODIPY TR and also new mounting methods as well as imaging alternatives for the whole brain in early stages, with the aim of contributing to zebrafish digital atlases and databases.

Keywords: Development; Neuronal Circuits; Anatomical Characterization; Zebrafish

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Abbreviations List

Cer – Cerebellum

Ha – Habenula

MLF – Medio Longitudinal Fasciculus

MO – Medulla Oblongata

nMLF – Nucleus of MLF

OMn – Oculomotor nucleus

Oe – Olfactory Epithelium

TeOn – Optic Tectum Neuropil

TeOsp – Optic Tectum Stratum Periventriculare

OP – Otic Placode

Pc – Posterior commissure

pLL – Posterior Lateral Line Ganglia

Pr – Pretectum

pTha – Prethalamus

Ret – Retinal layer

Teg – Tegmentum

Tha – Thalamus

TG – Trigeminal Ganglia

VG – Vagal Ganglia

Vagus MN – Vagus Motor Neurons

vcc – Ventro-caudal Cluster

1. Introduction

1.1. Zebrafish as a model

The nervous system is one of the most fascinating structures to study but one of the least understood. It controls function and behavior of organisms, by integrating internal and external stimuli, interpreting it and responding to them in concordance. Since it plays such an important role, the molecular pathways, involved in its development are very conserved among vertebrates.

The zebrafish (*Danio rerio*) is a freshwater fish belonging to the cyprinidae family. This fish is native to South Asia, usually inhabiting slow moving to stagnant shallow waters in streams and ponds.

Due to its biological characteristics, the zebrafish has become one of the most known animal models in scientific research. Research with this model is widespread in many fields of science, yielding advances from genetics, oncology, stem cells research, developmental biology to neuroscience[1]. Furthermore, accordingly to Howe et al., 2013[1], zebrafish's genome is conserved among other animal models such as the mouse (*Mus musculus*) and the chicken (*Gallus gallus*), sharing between these and human, more than 10 000 genes. Along with this, it's simplicity and wide availability of powerful genetic tools allows the generation of stable transgenic lines, that can be further studied.

Adding to these interesting attributes, the biological characteristics of this fish species makes it a suitable model for neuroscience. The embryos and larvae have a natural transparency, that allows imaging the whole brain *in vivo*. In addition, brain development takes place in a short period of time. In fact, after one week of development, the zebrafish brain presents the same anatomic pattern of most vertebrates and also a set of innate behaviors.

Knowing that zebrafish is an oviparous fish, the set of innate behaviors related to vision, at early developmental stages, is fundamental to its survival. Vision provides a suite of cues from the environment allowing adaptative behavioral responses. These larvae show reflexive eye movements when following a rotational motion stimulus (optokinetic response) and adaptation of the swimming behavior to the perceived water movements (optomotor response)[2].

All reasons listed above make evident that zebrafish larvae are the ideal model to study neuronal circuits, specially – on this dissertation – the ones related with visually guided behaviors.

1.2. Development of the zebrafish brain

During the nervous system development, cell fates are specified and organized in cell populations, promoting the acquisition of shape. This process - that culminates in a functional system - has many steps such as neural induction, regionalization, neurogenesis, neuronal differentiation, axonogenesis, synaptogenesis and synaptic remodeling[3,4].

The development of the zebrafish is known to be fast: after 5 hours post fertilization (hpf), it achieves the gastrula stage[5]. In this stage, the primary germ layers (endoderm, mesoderm and ectoderm) are formed as well as the embryonic axis[4].

Neural induction is the first step in development of the nervous system. The organizer (also known as Spemann organizer and Hensen's nod in frog and chicken models, respectively) induces part of the ectoderm into a neuronal fate. This is achieved with a combination of activators and inhibitors, that can be extrinsic (e.g. BMP, Wnt and Fgf secreted by the presumptive mesoderm)[6,7] or intrinsic (e.g. Sox family of transcription factors)[8] to this layer. Presumptive neural cells, in the neural plate, grow through the rostral-caudal axis along the dorsal midline.

Subsequently, the neuroectoderm, through neurulation, is rearranged into a neural tube. Even though, in zebrafish the neuronal tube is not formed as most vertebrates, the topological arrangement is still analogous. Primary neurulation starts with the formation of a solid neural keel that develops into a neural rod. The (usually called) secondary neurulation involves the rearrangement of the cells creating a lumen – neurocoele –, that will give rise to the neural tube[5,9]. During neurulation, the neural keel undergoes regional morphogenesis, creating 10 neuromeres (distinct swellings)[4,5]. The first 3 neuromeres will compose the forebrain (presumptive diencephalon and telencephalon) and the midbrain (presumptive mesencephalon). The other 7 neuromeres are subdivisions of the hindbrain (presumptive rhombomeres 1-7). The cerebellum primordium can be seen at 19hpf near the hindbrain-midbrain boundary and after, at 22hpf, the hypothalamus and epiphysis, in the diencephalon, are recognizable[5].

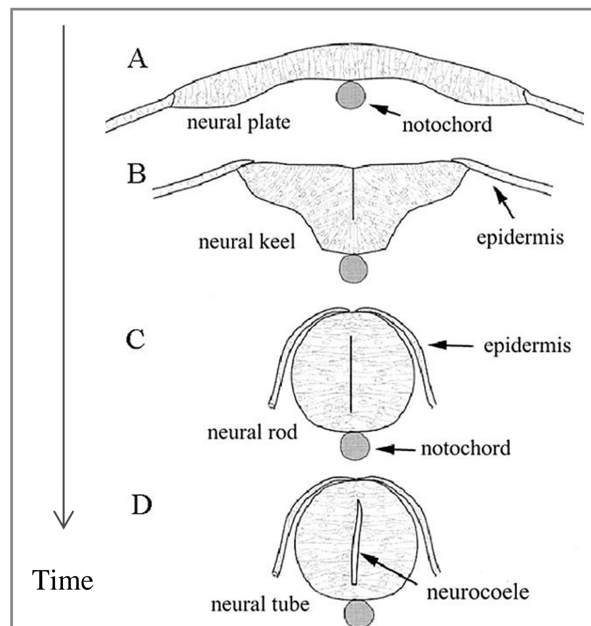


Figure 1.1 – **Development of the zebrafish neural tube through time.** A. Neural Plate. B. Neural Keel. C. Neural Rod. D. Neural Tube. Adapted from Geldmacher-Voss et al., 2003.

Neurogenesis in zebrafish is a process that comprises the induction, proliferation and differentiation of neural progenitor cells into mature neurons[10]. Embryonic neurogenesis starts during gastrulation with the expression of proneural genes[4,11]. These genes encode for transcription factors like the bHLH (basic Helix-Loop-Helix) class, determining neural progenitor cells like neuroepithelial cells[12]. In the neural tube, neuroepithelial cells can divide symmetrically, leading to a phase of proliferation and then divide asymmetrically starting differentiation.

Asymmetrical divisions occur through interkinetic nuclear migration[13], where the division takes place at the apical side and the differentiation at the basal side, establishing an apical-basal polarity. The fate of these cells is related to lateral inhibition[14] given by notch signaling considering that lower notch activity leads to differentiation and higher notch activity maintains a progenitor pool[15].

This process is classically named "primary neurogenesis" and creates a primary neuronal scaffold. The primary scaffold is composed of new-born neurons which axonogenesis starts between 14 and 24hpf[4]. The forebrain clusters extend axonal tracts connecting not only each cluster to one other but also to the spinal cord and peripheral nervous system. The medial longitudinal fasciculus (MLF) has also an important role in this scaffold since it contains axons of midbrain and hindbrain that connect to the spinal cord. Moreover, caudally, each rhombomere has reticulospinal neurons (long interneurons) that connect to the spinal cord[4].

The "secondary neurogenesis" expands and enhances the primary scaffold, even though there is no clear division in time or space of these phases. During this phase, neuromodulation and other regulatory systems are refined and established. For instance, secondary motor neurons create connections with the axial muscles by guiding their axonogenesis through the primary tracts. Contrary to primary motor neurons, secondary motor neurons have more control in muscle contraction producing finer movements[4].

Furthermore, proliferation and postmitotic neuronal differentiation is restricted to ventricular areas[4]. The regionalization of the neural tube (rostro-caudal and dorsal-ventral) provides paracrine gradients and different extracellular and cell signals that contribute to the further development of the different neuronal subtypes and glial cells. Additionally, neural activity and environmental factors help neural cells to differentiate and also mature over time[16].

The hatching period occurs spontaneously between 48 and 72hpf[5].

Thus, in the zebrafish, the first neurons are formed still during gastrulation, and as consequence embryos acquire spontaneous contractions at the end of the 1st day post fertilization (dpf)[17]. On the 2nd dpf zebrafish larvae, can respond to touch stimuli[17] and within a week already display a set of innate behaviors[2,18], such as visually guided behaviors (optokinetic and optomotor responses). Moreover, after one week, the zebrafish brain presents not only the same basic anatomic pattern of most vertebrates but also similar circuits[19], being an excellent model to study conserved pathways.

1.3. Visually Guided Behaviors in Zebrafish

Zebrafish presents, from early age, a set of innate behaviors. Visually guided behaviors, such as optokinetic and optomotor response, are behaviors well established allowing to further study the circuits behind it. Understanding how activity in the brain induces this kind of behavior is a great challenge in neuroscience.

1.3.1. Optokinetic Response

The optokinetic response (OKR) consists in reflexive eye movement that allows the organism to follow a rotational motion stimulus[2]. This behavior is spread across vertebrates, and it is widely used in zebrafish behavior studies, due to its consistency after 72hpf [20].

The OKR is characterized by two specific eye movements. One is often called slow phase and results on the eye movement following the rotational motion. The other is called fast phase (or optokinetic nystagmus) and it's the movement that resets the eye to its original position[21].

This circuit is known to receive an input to the contralateral pretectum, going directly to the abducens nucleus (ABN) or indirectly via the velocity storage mechanism (vsm) and via velocity to position neural integrator (vpni) to the ABN (*Figure 1.1*). From this structure, a motor output is sent to both eyes via the lateral rectus muscle (ipsilateral) and the medial rectus muscle (contralateral)[22].

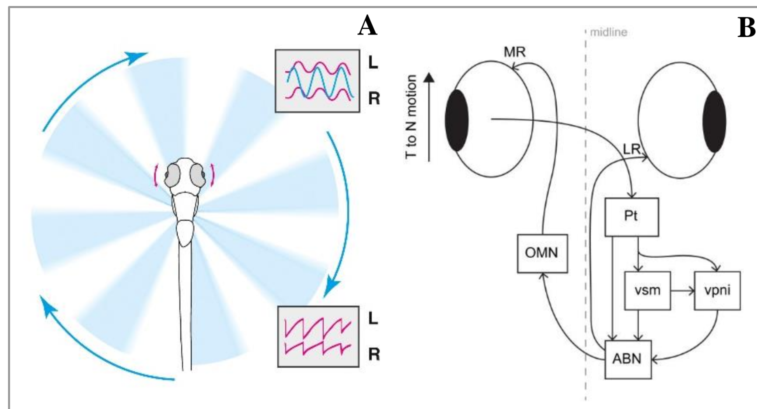


Figure 1.1 - **Optokinetic response in zebrafish.** A - Eye movements are induced by the rotational motion. Adapted from Orger, 2016. B – Proposed OKR circuitry. Adapted from Portugues et al., 2014.

1.3.2. Optomotor Response

The optomotor response (OMR) in zebrafish is characterized by the adaptation of the swimming behavior to a stimulus that resembles moving water. The larvae or fish will respond by orienting themselves to the perceived whole-field motion[2]. This behavior is innate and it's fundamental to correct a route that has suffered a perturbation.

The OMR circuitry receives input from the retina and the olfactory bulb. Each of these structures pass information to the pretectum and to the mesencephalic locomotor region that finally integrate it in the

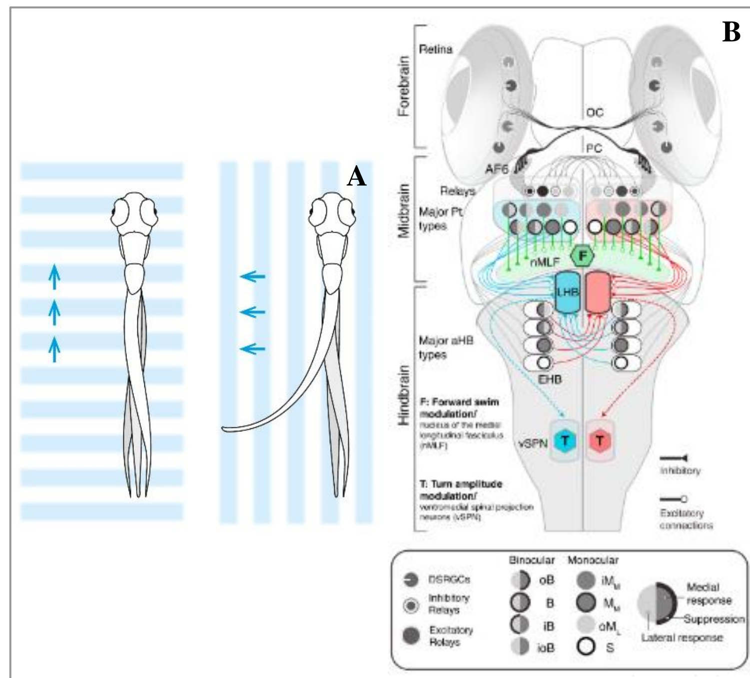


Figure 1.2 - **Optomotor response in zebrafish.** A - Re-orientation of the swimming direction is induced by whole-field motion. Adapted from Orger, 2016. B – Proposed OMR circuitry. Adapted from Naumann et al., 2016.

nucleus MLF. The nucleus MLF will pass this processed information for direct locomotion through the spinal cord (*Figure 1.2*)[23].

1.4. Transgenic Lines

Behind these behaviors, there are complex and difficult-to-access neuronal networks. Recently, with the advances in transgenic lines there are already tools that allow the manipulation of these circuits and the visualization of small populations of neurons at a time. Thus, one approach to the study of behaviors and their circuitry is through the construction and examination of transgenic lines. These lines encompass genes of interest and reporters, which make it possible to study in real time the response of a neuronal network's population to a stimulus (e.g. visual stimulus).

Some transgenic lines in zebrafish are generated through the GAL4-UAS system, or a derivative (GFF-UAS)[24]. This system allows the insertion of a gene of interest linked to the GFF portion and a reporter linked to the UAS portion. Depending on the gene or regulatory region of that gene of interest, lines can be pan neuronal – expressing the reporter throughout the brain – or restricted – expressing the reporter only in specific subpopulations. While pan neuronal transgenic lines are used more broadly and usually for counter staining, transgenic lines with more restricted expression can give further insights of neuronal subpopulations.

This system can be introduced at the one cell stage in the zebrafish model by various methods, being the most common the transposon mediated transgenesis[25]. Therefore, by this method it is possible to inject a BAC (Bacterial Artificial Chromosome) containing the gene of interest and a GAL4 derivative (GFF) into the starting codon of the gene of interest or to inject a genetic insert directly. This method grants long term reliability of the lines and efficient transmission to the germ line. These lines are then kept and crossed with UAS lines that have a reporter, like GFP (Green Fluorescent Protein).

This project is done in collaboration with the Michael Orger Lab (Champalimaud Foundation), where several transgenic lines are being created and studied. Studies in this lab focus on the visualization *in vivo* of the responses of neuronal network's subpopulation to a given stimulus. For this, the transgenic lines used are often associated with the UAS-GcaMP6. As result, numerous neuronal subpopulations that respond to stimuli such as light intensity and change of direction during movement have been identified (Renninger and Orger, unpublished).

These populations are then characterized anatomically to assess their location within specific brain regions and their identity, with the help of the literature and atlases. The transgenic lines used on this project have already been examined at the 6dpf larval stage (Renninger and Orger, unpublished), but not at early stages of development.

To further characterize some of these lines - currently in study at the Orger Lab - in this project we used the following transgenic lines:

- Tg(Pitx2c:GFF) (S. Renninger, R. Tomás and M. Orger, in preparation) – The *pitx2c* gene (paired-like homeodomain transcription factor 2) is known to play an important role in the development of the eye and in determining the left-right asymmetry in the diencephalon[26,27]. It has also been described that it might have some importance in transmitting locomotor information, since there is expression of this gene in nucleus of the medio longitudinal fascicle MLF (nMLF)[28].

- TgBAC(sst1:GFF line) (S. Renninger, R. Tomás and M. Orger, in preparation) – The *sst1* gene (somatostatin 1) is known to regulate cell migration through cell communication, having an impact in the preoptic area, hypothalamus and pituitary[29].

Other 2 transgenic lines were used in this project:

- lx200 line - This is a line (S. Renninger and M. Orger, in preparation) that was previously characterized during development[30], and it is being used here as guideline for the second section of this project - Establishing a Reference Marker and Sample Orientation for the Generation of a Brain Atlas for the 2dpf Zebrafish Embryo.

1.5. Anatomical Characterization

In order to understand how the brain controls animal behavior, it is important to identify the circuits behind that behavior and consequently assign brain regions to each circuit. This process, which allows each cell to be assigned to a region in the brain, is classified as anatomical characterization.

Several extensive atlases have been developed recently corresponding to the 6dpf larval brain. Open source digital atlases – like the Z-Brain[31] and the Zebrafish Brain Browser (ZBB)[32] – consist of a collection of 3D images of a large number of reporter lines and neuronal markers registered to a common reference brain as well as anatomical labels to annotate the different well known brain regions. In the case of the Z-Brain atlas, brains were immunostained to detect the tERK pan neuronal protein to register to the corresponding reference brain, while in the case of the ZBB[32] a broadly expressed mCherry line was used as the reference. Furthermore, a recent atlas – Max-Planck Zebrafish Brain Atlas[33] – was generated using immunohistochemistry to detect pan neuronal proteins such as tERK, SYP and HuC/D, in order to align their single-neuron labelled brains into the reference brain. Similar although different registration algorithms were used in each case.

For the earlier stages, a large body of information on brain anatomy is available although dispersed in numerous articles[26–29,34,35]. However, only partial attempts have been described to date to provide a more comprehensive atlas. The ViBE-Z atlas[36] described a collection of 4dpf zebrafish brains alongside the annotated brain regions but this is not currently accessible online. The Atlas of Early Zebrafish Brain Development[3] is a 2D atlas that uses *in situ* hybridization and immunohistochemistry to show the location of some well-known fundamental genes during primary neurogenesis in zebrafish and also phenotypic markers, focusing on the 2, 3 and 5 dpf. Additionally, efforts to compile an early atlas have been reported from the Wilson lab (www.zebrafishbrain.org/).

These are powerful and enriched tools that have been used in the field. Nevertheless, a digital atlas with a similar characterization for early stages of the zebrafish development is still necessary. Knowing how the brain develops by characterizing the expression of reporter lines, will provide insights of how the circuitries are built. This will play a major role in orienting new experiments and in understanding behavior, especially the visually guided behaviors.

In this project, we are addressing the characterization of zebrafish transgenic lines that have been generated in the Michael Orger lab and that allow the labelling of interesting neuronal subpopulations presumably involved in visually guided behaviors. Our goal is to determine where these neuronal

populations are located within the developing brain from the 1st dpf to the 6th dpf. To do this task, we have resorted to all listed atlases, and compared and compiled the information given.

Currently no 3D atlases have been developed for early stages. In this way, brain images cannot be included within a common reference brain and therefore assignment of brain regions have to be done by visual side by side comparison. To annotate the brain, conspicuous landmarks, such as commissures and ganglia, will be used.

However, a long-term goal in the laboratory is the generation of a 3D atlas at different stages in development. For that we need to identify a useful counter staining method to use as a reference for image registration and to establish the proper orientation of the samples that at the earlier stages are not as easily mounted as the 6dpf larvae. We address these topics in the second section of this project - Establishing a Reference Marker and Sample Orientation for the Generation of a Brain Atlas for the 2dpf Zebrafish Embryo.

As possible stains we set to examine:

- tERK staining – which is the reference counter staining used in the 6dpf Z-Brain and ZBB atlases[31,32,37].

- alphaTubulin:mScarlet[38] – a transgenic line that has the gene encoding the monomeric fluorescent protein mScarlet under the control of the pan neuronal *alpha-1-Tubulin* gene regulatory region.

- DiD[39] – a far-red fluorescent dye that stains cell membranes and lipidic vesicles.

- BODIPY TR methyl ester[40]. – a synthetic red fluorophore that stains endomembranous organelles.

For the orientation of the embryo we tried to image the whole embryo brain by reorienting it while mounting. Since there is a curvature between the forebrain and midbrain, embryos in early stages tend to have only part of the brain imaged through conventional methods. To overcome this issue, we took advantage of new mounting techniques and different equipment (confocal and light sheet microscopes).

1.6. Aims of this thesis

Since there are no 3D atlases of the zebrafish brain at early stages, we aim to characterize two transgenic lines through development and also improve the imaging conditions for this developmental characterization. Therefore, this thesis is divided in two main sections:

- Anatomical Characterization of the Pitx2c:GFF and the sst1:GFF Transgenic Lines (Orger lab) Through Development

- Establishment of a Reference Marker and Sample Orientation for the Generation of a Brain Atlas for the 2dpf Zebrafish Embryo

The first section focuses on the characterization of neuronal subpopulations linked to visually guided behaviors. There, two transgenic lines are going to be thoroughly analyzed, from the 1st to the 6th dpf.

Their GFP expression pattern is going to be examined, resulting in an anatomical annotation that will in the end be compared to other atlases and bibliography.

The second section is going to be a study on two major challenges in the anatomical characterization process. The first challenge is related to the counter staining. Here we will compare different available counter staining methods, from antibodies to dyes and transgenes. The second challenge directs to the orientation of embryos (in early stages) while mounting. Since there is a curvature between the forebrain and midbrain, with conventional methods it is difficult to image the whole brain with the right resolution. Here we will compare not only the mounting alternatives, but also equipment (microscopes).

2. Materials and Methods

2.1 Animals and Transgenic Lines

2.1.1. Fish Maintenance

The welfare of the zebrafish used was provided by the vivarium platform at the Champalimaud Foundation[41]. The vivarium rooms were maintained at a controlled temperature (25ºC), humidity (50% - 60%) and light/dark cycle (14h light/10h dark). Fish were housed in 3.5 l tanks and fed with rotifers, artemia and dry food daily, depending on their stage of development and maturation. The light intensity at the water surface of the tanks is 200-300lux. All physiological conditions of the water (pH, salinity and dissolved gases) were monitored continuously through the system and checked daily.

2.1.2. Breeding and collection of the embryos

For breeding purposes, fish were placed in breeding tanks which are specialized tanks with a slanted insert and a removable divider. Females and males were placed inside the breeding tank in a ratio of 5♀:2♂ and were kept separated by the divider. The tanks were left overnight and early in the next morning the divider was taken out. After approximately 1h and 30 min, the eggs were collected and incubated in E3 medium (*See Solutions*) at 28.5ºC with a density of about 50 eggs per petri dish (100x15mm).

On the 1st dpf the non-developing embryos are discarded. The petri dish is changed to avoid the propagation of microorganisms and the medium is replaced by E3+PTU (*See Solutions*) to avoid the development of pigmentation. This medium is then changed every two days until the 6dpf, when larvae must be sacrificed. Between the 1st dpf and the 6th dpf, living embryos and larvae can be discarded in bleach for euthanasia.

All experiments with animals were done according to the CF Animal Welfare Body guidelines and under a CF Ethics Committee-approved project.

2.1.3. Transgenic Lines Used

The GFF expressing transgenic lines selected are used in the Michael Orger Lab to study visually guided behaviors and have been previously generated (Renninger and Orger, unpublished), using the tol2 transposon system[25] for recombinant DNA integration.

The sst1:GFF line was generated by injection of a construct including a Bacterial Artificial Chromosome (BAC) containing a the *sst1* gene (in addition to surrounding genomic regions and genes) and has been modified inserting a GAL4 derivative (GFF) at the starting ATG of the gene of interest. This approach aimed at expressing GFF in *sst1*$^{+}$ cells and the name "sst1:GFF" was maintained for it throughout this thesis, having also the aim to confirm whether it labels the expected neurons.

A similar approach but with a BAC including the *ChAT* gene was previously generated and is currently named "lx200" as it does not appear to label the *ChAT*$^{+}$ cells.

On the other hand, the Pitx2c:GFF line was created by injecting a DNA construct including a genomic fragment from the *pitx2c* regulatory region upstream of the GFF coding region.

These GFF expressing fish are maintained in the laboratory in a UAS:LynmCherry or UAS:mCherry background and here, were crossed with UAS:GFP fish[24], in order to express GFP to visualize the specific GFF expressing cell populations of each line.

Another line used in this project was the alphaTubulin:mScarlet, created by injection of plasmid containing the gene encoding the protein mScarlet under the control of the alpha-1-Tubulin promotor[38].

2.2. Sample preparation and Immunohistochemistry

2.2.1. Screening of the embryos

Zebrafish embryos and larvae were screened for GFP expression under a fluorescence dissecting microscope (Zeiss Stereo Lumar V.12), before the fixation step. The specimens with the best fluorescence expression of the reporter transgenes were selected to continue the process.

Screening of green and red fluorescence signal is essential to select the correct phenotypes – either GFP expressing cells only or GFP and mScarlet expressing cells –, as required for each experiment. Screening is also used to ensure that the fish used do not carry other transgenes (i.e. LynmCherry) that result in the expression of other fluorescence proteins and that are present in the GFF parental line for maintenance.

2.2.2. Fixation of the embryos and larvae

After screening, embryos and larvae were kept at 28.5ºC until the right time point for fixation. Before fixation embryos and larvae were anesthetized with tricaine 1x and then sacrificed with tricaine 25x (*See Solutions*). Fish were then washed in PBT and fixed overnight in PFA at 4ºC (*See Solutions*).

After fixation samples were washed in PBT and stored at 4°C in PBT with 0,05% of Sodium Azide until the immunohistochemistry protocol was continued.

2.2.3. Immunohistochemistry

For immunofluorescence staining of the whole embryo and larvae, we followed a protocol adapted from Randlett et al., 2015[31]. For epitope retrieval, samples were incubated in 150 mM Tris-HCl pH9, for 15 min at 70ºC and then rinsed and washed in PBT. For permeabilization of cell membranes, larvae from 4 dpf to 6 dpf, were incubated in 0.05% trypsin-EDTA for 5 min on ice and then rinsed and washed again in PBT. The blocking buffer was then applied with rotation.

After 2h to 3h the samples were incubated in a solution containing the primary antibodies (*See Antibodies*) for 3 days with mild shaking at 4ºC. Samples were then rinsed and washed in PBT and incubated with the secondary antibodies for 3 days with mild shaking at 4ºC. After this procedure, embryos and larvae were rinsed and washed in PBT and stored with 0,05% of Sodium Azide at 4ºC.

2.2.4. Antibodies

The samples were incubated with primary and secondary antibodies in AB mix (*See Solutions*). The primary antibodies used were anti-GFP (generated in chicken) and anti-tERK (generated in mouse). As

secondary antibodies we used anti-chicken coupled to Alexa488 and anti-mouse coupled to Alexa633. All antibodies were diluted (1/500 µl) for incubation.

Table 2.1 - **List of antibodies used.** Antibodies used and their manufacturer reference.

Antigen	Manufacturer Reference	Host/ Isotype	RRID
GFP	Abcam: ab13970	Chicken/ IgY	AB_300798
tERK (total p44/42 map kinase)	Cell Signaling: 4696S	Mouse/ IgG1	AB_390780
Chicken IgY (H+L)	ThermoFisher: A-11039	Goat/ IgG	AB_2534096
Mouse IgG (H+L)	ThermoFisher: A-21052	Goat/ IgG	AB_2535719

2.2.5. Dyes

After immunohistochemistry, some samples were stained with dyes in order to have a different counter staining from the reference one (tERK staining). On these samples, instead of using the anti-tERK antibody, DiD or BODIPY TR methyl ester were applied.

DiD was used accordingly Affaticati et al., 2018[39], applying the staining solution of 1µM DiD during 5 days.

BODIPY TR methyl ester was applied in two different ways: in living samples accordingly Cooper et al., 2005[40] and in fixed samples with an alternative protocol. While in the first protocol BODIPY TR is applied *in vivo* and only after, the embryos are fixed, the second protocol is done by fixing the samples, submitting them to the immunohistochemistry step and only after this step, BODIPY TR is applied. Vital staining is applied for 1h at room temperature with a concentration of 100µM BODYPI TR. Fixed staining uses the same staining solution (with 100µM BODPI TR concentration) but it is applied for 5 days at 4ºC.

After this procedure, embryos and larvae were rinsed and washed in PBT and stored with 0,05% of Sodium Azide at 4ºC.

Table 2.2 - **List of dyes used**. Dyes used and their manufacturer reference.

Dye	Manufacturer Reference
DiD	Biotium (VWR): 60014
BODIPY TR Methyl Ester	ThermoFisher: C34556

2.3. Microscopy and Image Analysis

2.3.1. Mounting – Confocal Microscope

For confocal imaging the samples were mounted in low-melting agarose (1,5% in PBS). Each embryo or larva was mounted with their dorsal side directly in contact with the coverslip (#1.5), standing parallelly to the coverslip (*Figure 2.1.A*). After letting the agarose cool down to gel, a margin of silicone grease was put to surround the drop of agarose, creating a space to insert PBS in order to avoid the sample to dehydrate. This is then gently pressed against the slide creating a watertight seal.

Some embryos were mounted "vertically" – with the rostral part of the head in direct contact with the coverslip – creating a 90º angle of the dorsal side with the coverslip (*Figure 2.1.B*).

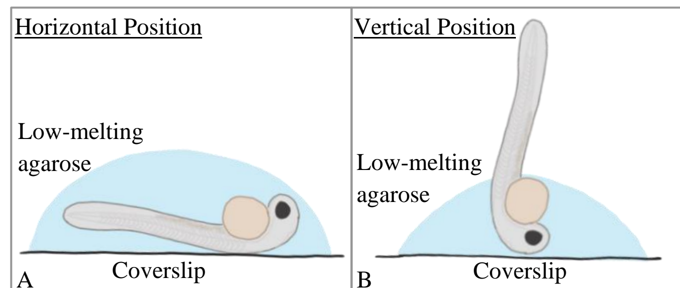


Figure 2.1 - **Schematic of mounting methods used with 2dpf embryos for confocal imaging**. A. Horizontal position: embryo is mounted with its dorsal side directly in contact with the coverslip, lying parallelly to it. B. Vertical Position: embryo is mounted with its rostral part of the head in direct contact with the coverslip (a 90º angle is created between the dorsal side and the coverslip. The schematic is not on scale.

2.3.2. Mounting – Light Sheet Microscope

For light sheet imaging, embryos were mounted in a capillary with low-melting agarose (0.8% in PBS). The embryos were mounted as if lying on their side, in a way that one eye was pointing up and the other was pointing down with their dorsal part close to the capillary (*Figure 2.2*).

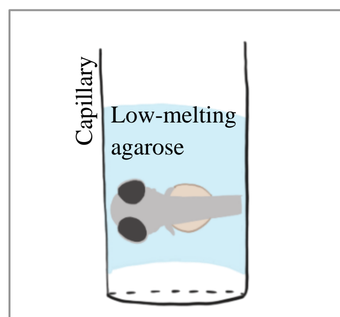


Figure 2.2 - **Schematic of the mounting method used with 2dpf embryos for light sheet imaging**. Embryos were mounted facing the capillary wall in a way on eye was pointing up and the other was pointing down. The schematic is not on scale.

2.3.3. Imaging acquisition – Confocal Microscope

With the upright confocal laser point-scanning microscope - Zeiss LSM 710 - samples were imaged (16-bit images) through a 25x multi- immersion objective (N.A. 0.8), using a combination of lasers (Argon: 488 nm (for GFP), DPSS: 561 nm (for mScarlet and BODIPY TR methyl ester) and HeNe633: 633 nm (for anti-tERK and DiD). Water was used as immersion medium.

Parameters like power of the laser, dwell time, digital gain and offset were optimized through the ZEN software to avoid saturation and adjust signal levels. The voxel size used was 0.8 µm × 0.8 µm × 1 µm (x × y × z).

For early stages (1-2 dpf) one tile could cover the area of interest while for later stages (3 to 6 dpf), the whole brain was imaged through 10% overlapping tiles that were stitched together in Fiji (*See Image Analysis*).

2.3.4. Imaging Acquisition – Light Sheet Microscope

With the light sheet fluorescence microscope - Zeiss Light sheet Z.1- samples were imaged through a 20x multi-immersion objective (N.A. 1.0), using 10x illumination dry lenses (N.A. 0.5), a combination of solid state laser (488 and 561) and a sCMOS camera (pco.edge 5.5). The chamber was filled with PBS or PBT, since the embryos imaged were already fixed. The voxel size used was 0.25 µm × 0.25 µm × 0.5 µm (x × y × z).

2.3.5. Image Analysis

Image analysis was done through Fiji[42]. Images with two tiles went through a pairwise stitching process to create a whole brain z-stack. To help in identification of brain regions, reslicing and orthogonal views were used. These brain images were then compared between each stage, and the representative brains were chosen for the comparison with bibliography.

Currently no 3D atlases have been developed for early stages. This will be an attempt to assign brain regions using only visual comparison (side by side) to bibliography. In this way, annotation was based on prominent landmarks (e.g. commissures and ganglia). The bibliography used for comparison was reference brains, reference stacks and images from Z-brain[31], zbb atlas[32] and Muller and Wullimann[3].

Here, the best embryo or larva was chosen to represent each line and stage, in the format of a z-projection, anatomically identified. At least 3 samples per condition were analyzed.

For the second section of this project – Establishing a Reference Marker and Sample Orientation for the Generation of a Brain Atlas for the 2dpf Zebrafish Embryo – an attempt to make a qualitive comparison between staining alternatives and orientation procedures and equipment were performed.

2.4. Solutions

All solutions used, are listed in *Table 2.3*.

Table 2.3 - **Solutions Table**. Composition of all solutions

Solution	Composition
E3	5mM NaCl, 0.17mM KCl, 0.33mM CaCl2, 0.33mM MgS04, pH=7.2
PBT	0.25% of Triton in PBS
Tricaine (25X)	1.6 mg/mL
PFA	4% of Paraformaldehyde in PBT
PBT	0.25% of Triton in PBS
TRIS-HCL	150 mM, pH 9
Trypsin-EDTA	10% of Trypsin-EDTA at 0.5% and 10% of PBS 10x in H_2O
Blocking Buffer	1% of BSA (Bovine Serum Albumin), 2% of NGS (Normal Goat Serum), 1% of DMSO (Dimethyl Sulfoxide) in PBT
Primary Antibody Mix	All primary antibodies were diluted (1/500) in PBT containing 1% BSA and 1% DMSO
Secondary Antibody Mix	All secondary antibodies diluted (1/500) in PBT containing 1% BSA and 1% DMSO
E3+PTU	PTU (0.2 mM) in E_3
Sodium Azide	0.05% Sodium Azide

3. Anatomical Characterization of Transgenic Lines Through Development

3.1. Results

3.1.1. Characterization of the Pitx2c:GFF line

The *pitx2c* gene (paired-like homeodomain transcription factor 2) is known to play an important role in the development of the eye and in determining the left-right asymmetry in the diencephalon, specifically in the habenula[26,27]. It has been previously described[28] that, in early stages, there is expression of the *pitx2c* gene in the nucleus of the MLF (nMLF), being the nMLF the primary way to transmit locomotor information.

These structures - the eye, habenula and the nMLF – are associated with visually guided behaviors, making this transgenic line ((pitx2c:GFF);UAS:lyn-mcherry) suitable to study this behavior.

3.1.1.1. The Pitx2c:GFF line at the 1dpf stage

At 1 dpf, GFP expression is present in a few cells and at low levels in the mesencephalon. This region is probably part of the ventro-caudal cluster (vcc).

In the periphery, there are two clusters of GFP expression on both sides, that are part of the otic placode (OP) (*Figure 3.1*).

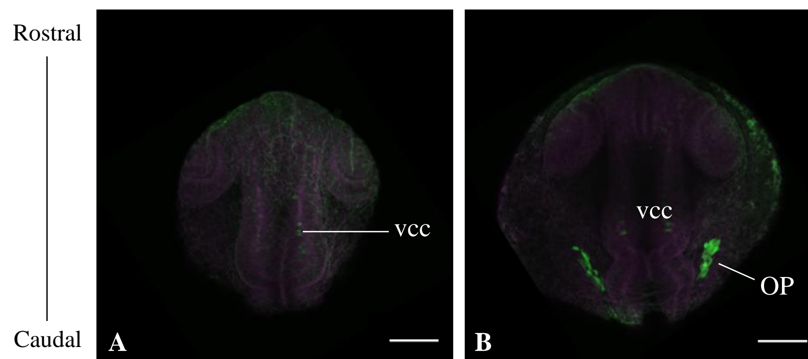


Figure 3.1 – **Pitx2c: GFF Expression in the 1dpf embryos**. A-B, z-projection of the 1 dpf brain of the Pitx2c:GFF line from dorsal (A) or ventral (B) regions after immunohistochemistry with anti-tERK (magenta) and anti-GFP (green) antibodies. The scale bar indicates 100 µm. Abbreviations: OP – otic placode, vcc – ventral-caudal cluster. Four samples were analyzed.

3.1.1.2. The Pitx2c:GFF line at the 2dpf stage

At 2dpf, GFP expression is still low. In the mesencephalon, GFP expression is present in the ventro-caudal cluster (vcc), in the region that is going to become the medulla oblongata (MO) and in the otic placode (OP) (*Figure 3.2.A-B*).

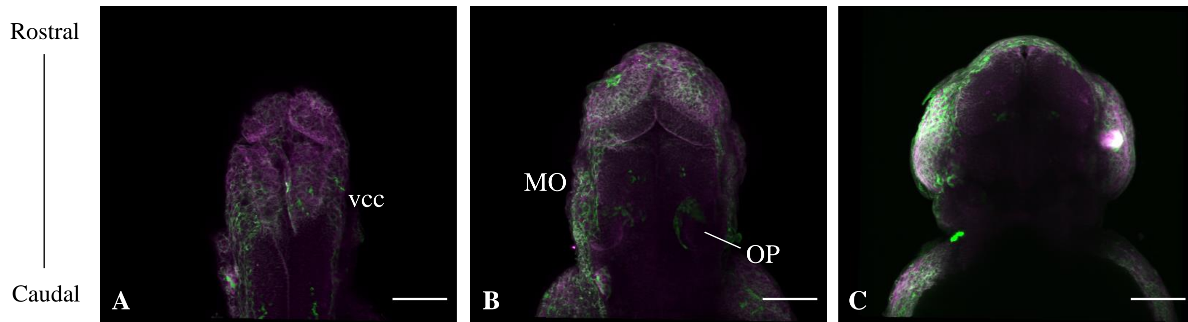


Figure 3.2 – **Pitx2c: GFF Expression in the 2 dpf embryos**. A-C, z-projection of the 2 dpf brain of the Pitx2c line from dorsal (A) to ventral (C) regions after immunohistochemistry with anti-tERK (magenta) and anti-GFP (green) antibodies. The scale bar indicates 100 µm. Abbreviations: OP – otic placode, MO – medulla oblongata, vcc – ventral-caudal cluster. Four samples were analyzed.

3.1.1.3. The Pitx2c:GFF line at the 3dpf stage

At 3dpf, GFP expression is observed in cells distributed throughout the whole brain.

In the telencephalon, there is expression of GFP in the olfactory epithelium (Oe) (*Figure 3.3.D*).

In the diencephalon, there is GFP expression in the pretectum (Pr) (*Figure 3.3.B*), in the thalamus (Tha) (*Figure 3.3.C*) and in the habenula. Habenular asymmetry is present, showing GFP expression only in the left habenula (lHa) (*Figure 3.3.A*).

In the mesencephalon, dorsally, the optic tectum neuropil (TeOn) and the optic tectum stratum periventriculare (TeOsp) present GFP expression (*Figure 3.3.A*). Additionally, in more ventral brain regions, there is expression in part of the tegmentum (Teg) - especially in the nucleus of MLF (nMLF) and in the oculomotor nucleus (OMn) (*Figure 3.3.B-C*).

In the metencephalon, GFP expression in the caudal part of the cerebellum (cCer) can be observed *(Figure 3.3.A)*.

In the myelencephalon, the posterior lateral line ganglia (pLL) can be observed (*Figure 3.3.B* for the right pLL; *Figure 3.3.C* for the left pLL). More ventrally, and bellow the posterior lateral line ganglia, there is expression in the vagal ganglia (VG) (*Figure 3.3.C* for the right VG; *Figure 3.3.D* for the left VG), and caudally, still within the myelencephalon, there is expression in the vagus motor neurons (Vagus MN) and in the medulla oblongata (MO) (*Figure 3.3*).

Trigeminal ganglia (TG), which are located at the periphery, have also strong GFP expression (*Figure 3.3.D*).

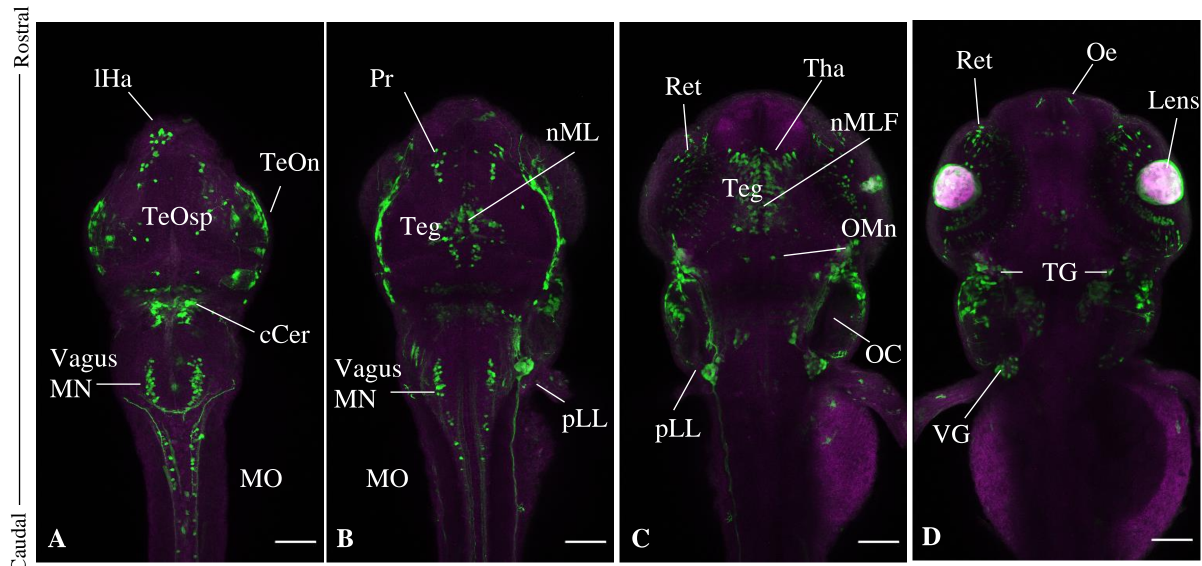


Figure 3.3 – **Pitx2c: GFF Expression in the 3 dpf embryos.** A-D, z-projection of the 3 dpf brain of the Pitx2c line from dorsal (A) to ventral (D) after immunohistochemistry with anti-tERK (magenta) and anti-GFP (green) antibodies. The scale bar indicates 100 µm. Abbreviations: cCer – caudal cerebellum, lHa – left habenula, Lens – lens, MO – medulla oblongata, nMLF – nucleus of the MLF, OC – otic capsule, Oe – olfactory epithelium, OMn – oculomotor nucleus, pLL – posterior lateral line ganglia, Pr – pretectum, Ret – retina, Teg – tegmentum, TeOn – optic tectum neuropil, TeOsp – optic tectum stratum periventriculare, TG – trigeminal ganglia, Tha – thalamus, Vagus MN – vagus motor neurons, VG – vagal ganglia. Five samples were analyzed.

3.1.1.4. The Pitx2c:GFF line at the 4, 5 and 6dpf stages

On the 4th, 5th and 6th dpf the expression of GFP is well defined. There is no change in the expression pattern established on the 3rd dpf, only an increase in the number of cells.

In the telencephalon, there is expression in the olfactory epithelium (Oe) (*Figure 3.4.D,H*).

In the diencephalon, there is GFP expression in the left habenula (lHa) (*Figure 3.4.A,E,I*), in the thalamus (Tha) (*Figure 3.4.C,G,K*) and in the pretectum (Pr) (*Figure 3.4.B,F,J*). The posterior commissure (pc) is also a structure expressing GFP, in the diencephalon (as shown in *Figure 3.4.B,F,J*). Note that in the habenula the expression is more prominent on the left side, even though there are some GFP expressing cells on the right side.

In the mesencephalon, the optic tectum neuropil (TeOn) and the optic tectum stratum periventriculare (TeOsp) can be observed with GFP expression (*Figure 3.4.A,E,I*). The tegmentum (Teg) has GFP expressing cells, in particular, in nucleus of MLF (nMLF) and in the oculomotor nucleus (OMn) region (*Figure 3.4.B-C, F-G, J-K*).

In the metencephalon, there is GFP expression on the caudal part of the cerebellum (cCer) (*Figure 3.4.A-B, E-F, I*).

The vagus motor neurons (Vagus MN) and the medulla oblongata, both in the myelencephalon, have cells with GFP expression.

Peripherally to the central nervous system, there is GFP expression in the trigeminal ganglia (TG), and vagal ganglia (VG) (*Figure 3.4.D,H,L*). In the retina, there is GFP expression, probably, in the inner nuclear layer (*Figure 3.4.C-D, G-H, K-L*).

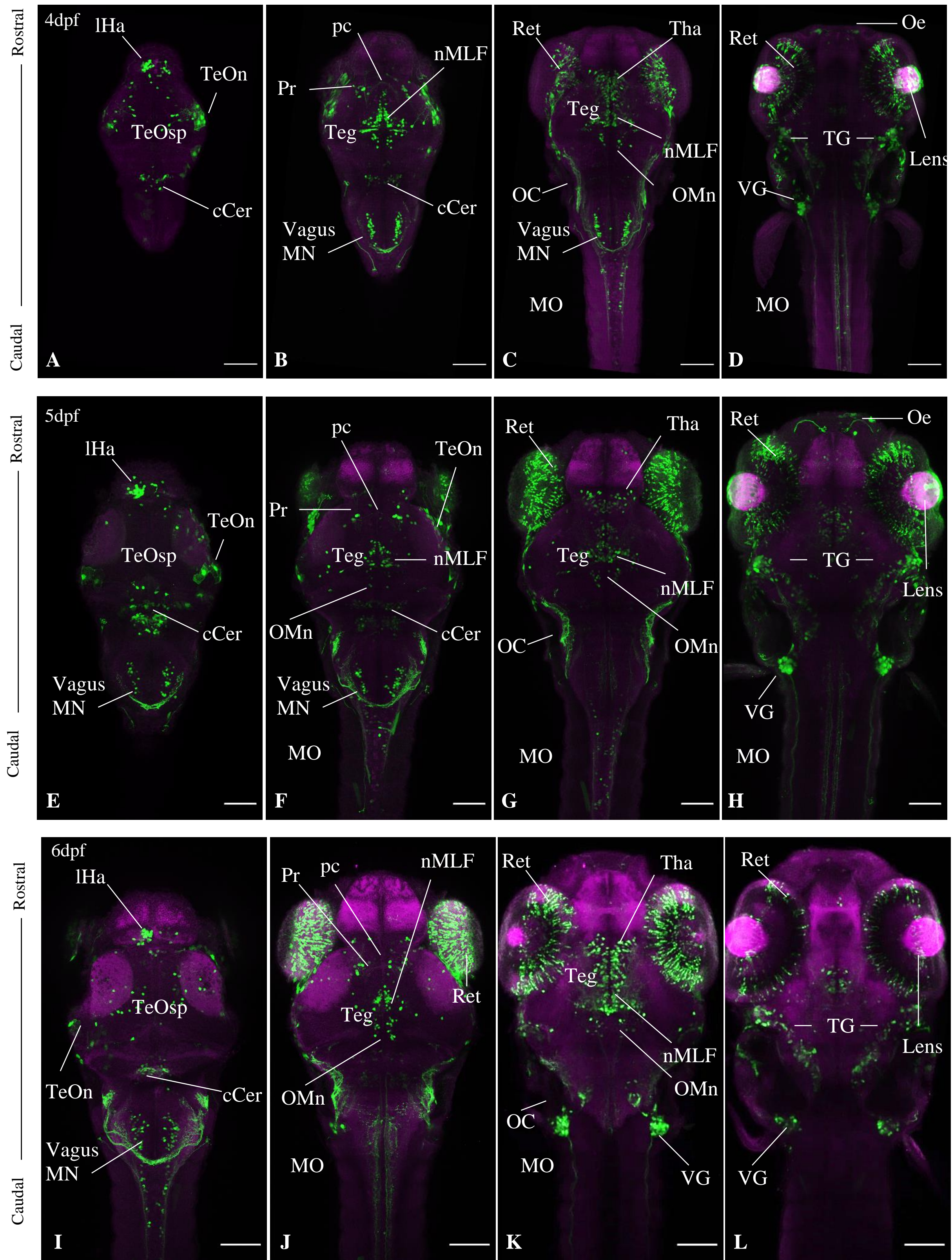


Figure 3.4 - **Pitx2c: GFF Expression in the 4, 5 and 6 dpf embryos.** A-D, z-projection of the 4 dpf brain. E-F, z-projection of the 5 dpf brain. I-L, z-projection of the 6 dpf brain. All these images are from the Pitx2c line after immunohistochemistry with anti-tERK (magenta) and anti-GFP (green) antibodies. From dorsal (left) to ventral (right) regions. The scale bar indicates 100 µm. Abbreviations: cCer – caudal cerebellum, lHa – left habenula, Lens – lens, MO – medulla oblongata, nMLF – nucleus of MLF, OC – otic capsule, Oe – olfactory epithelium, OMn – oculomotor nucleus, pc – posterior commissure, Pr – pretectum, Ret – retina, Teg – tegmentum, TeOn – optic tectum neuropil, TeOsp – optic tectum stratum periventriculare, TG – trigeminal ganglia, Tha – thalamus, Vagus MN – vagus motor neurons, VG – vagal ganglia. Six samples were analyzed for the 4dpf stage, three samples for the 5dpf and 5 samples for the 6dpf.

3.1.1.5. Pitx2c:GFF Overview

In the early stages of development (1 to 2dpf), cells express low levels of GFP. These are mostly present in the mesencephalon, presumably in the ventro-caudal cluster (vcc), and peripherally in the otic placode (OP).

From the 3rd dpf, GFP expression is observed in most brain regions in specific patterns.

In the telencephalon, the olfactory epithelium (Oe) has GFP expressing cells.

In the diencephalon, there is expression in the pretectum (Pr), thalamus (Tha) and in the habenula (Ha). An asymmetry in the GFP expression is found in the habenula, where the left part of the habenula (lHa) presents, consistently, GFP expressing cells, while the right part of the habenula (rHa) does not.

In the mesencephalon, there is expression in the optic tectum neuropil (TeOn) and in cells of the optic tectum stratum periventriculare (TeOsp). Continuing – from dorsal to ventral – GFP is expressed in part of the tegmentum (Teg) - specially on the nucleus of MLF (nMLF) and in the oculomotor nucleus (OMn).

Within the metencephalon, GFP expression is present in the caudal part of the cerebellum (cCer).

In the myelencephalon, it is possible to observe the GFP expressing cells in the vagus motor neurons (Vagus MN) and in the medulla oblongata (MO).

Peripherally to the central nervous system, there is GFP expression in the posterior lateral line ganglia (pLL), trigeminal ganglia (TG), vagal ganglia (VG) and in the retina, possibly, in the inner nuclear layer.

At later stages of development (4, 5 and 6dpf), the number of cells in the different domains increase but there is no change in the established pattern of GFP expression.

3.1.1.6. Pitx2c:GFF Expression Summary

In *Table 3.1* a summary of expression of the Pitx2c:GFF line is presented.

Table 3.1 - **Overview of Pitx2c expression.** The regions listed above are following primarily the Z-brain atlas, but the ZBB and the Mueller et al, 2016 atlases were also consulted. (* - the structure listed can be assigned to other brain regions in the different atlases).

Brain Region	Time Range (dpf)	Areas with Expression	Abbreviations
Telencephalon	3-5	Olfactory Epithelium	Oe
Diencephalon	3-6	Habenula (left)	lHa
	3-6	Thalamus	Tha
	3-6	Pretectum	Pr
Mesencephalon	1-2	Ventro-caudal Cluster	vcc
	3-6	Optic Tectum Neuropil	TeOn
	3-6	Optic Tectum Stratum Periventriculare	TeOsp
	3-6	Tegmentum	Teg
	3-6	Nucleus of MLF*	nMLF
	3-6	Oculomotor nucleus	OMn
Metencephalon	3-6	Cerebellum (caudal)	cCer
Myelencephalon	3-6	Vagus Motor Neurons	Vagus MN
	3-6	Medulla Oblongata	MO
Retina	3-6	Inner nuclear Layer	Ret
Other	1-2	Otic Placode	OP
	3-6	Trigeminal Ganglia	TG
	3	Posterior Lateral Line Ganglia	pLL
	3-6	Vagal Ganglia	VG

3.1.2. Characterization of the sst1:GFF line

The *sst1* gene (somatostatin 1) is known to be produced in the central and peripheral nervous system and regulate locomotor functions[29]. In this way, the sst1 transgenic line used here TgBAC(sst1:GFF) can help to understand better the development of the neuronal circuits linked to visually guided behaviors.

3.1.2.1. The sst1:GFF line at the 1dpf stage

On the 1st dpf, there is not a defined GFP expression pattern. There are some GFP expressing cells, that are not assigned yet (*Figure 3.5*).

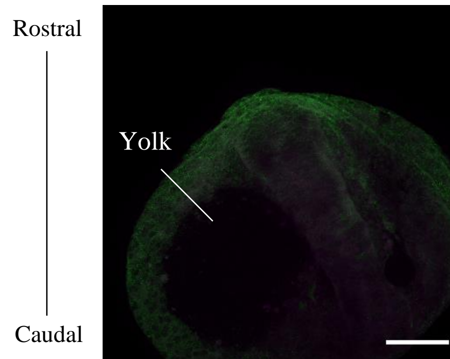


Figure 3.5 - **sst1: GFF Expression in the 1 dpf embryos.** z-projection of the 1 dpf brain of the sst1 line after immunohistochemistry with anti-tERK (magenta) and anti-GFP (green) antibodies. The scale bar indicates 100 µm. Three samples were analyzed.

3.1.2.2. The sst1:GFF line at the 2dpf stage

At the 2nd dpf, zebrafish embryos present expression in the mesencephalon, mostly in the optic tectum (TeO). There is also some GFP expression in the cerebellum (Cer), and more caudally in the myelencephalon, in the medulla oblongata (MO) (*Figure 3.6.A-B*). Only at this stage, it is possible to observe, ventrally, the stria medullaris (sm) formation (*Figure 3.6.C*).

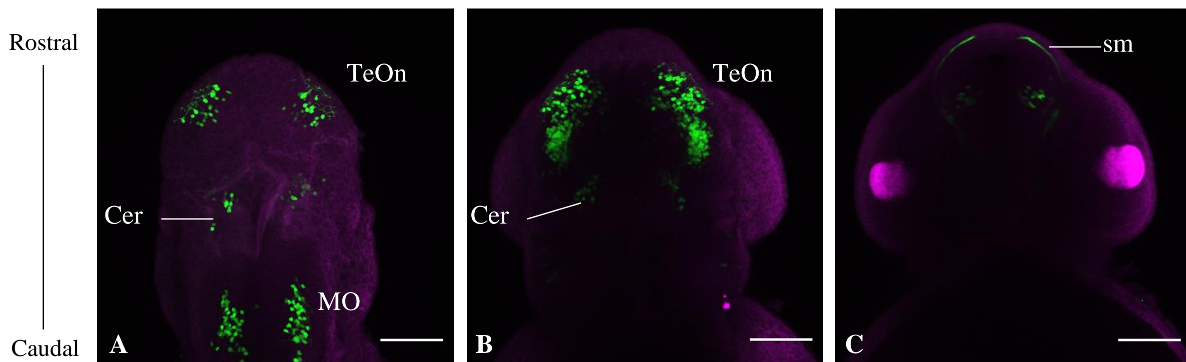


Figure 3.6 - **sst1: GFF Expression in the 2 dpf embryos.** A-C, z-projection of the 2 dpf brain of the sst1 line from dorsal (A) to ventral (C) after immunohistochemistry with anti-tERK (magenta) and anti-GFP (green) antibodies. The scale bar indicates 100 µm. Abbreviations: Cer – cerebellum, MO – medulla oblongata, TeO – optic tectum, sm – stria medullaris. Three samples were analyzed.

3.1.2.3. The sst1:GFF line at the 3dpf stage

At the 3rd dpf there is a cohesive pattern of GFP expression, that is going to be maintained (with some additions) on later stages.

In the telencephalon, the olfactory bulb (OB) has GFP expressing cells (*Figure 3.7.C-D*).

In the diencephalon, it is possible to observe the habenula (Ha), and the habenular commissure (HaC), that connects left and right habenula, as shown in *Figure 3.7.B*. Additionally, the posterior commissure (pc) starts to express GFP, at this stage (*Figure 3.7.B*).

The optic tectum stratum periventriculare (TeOsp) have GFP expression in the mesencephalon, and more ventrally on this region there is expression in the torus semicircularis (TS) (*Figure 3.7.A-B* and *Figure 3.7.C*, respectively).

The cerebellum (Cer), in the metencephalon, has GFP expression present.

There is GFP expression in the myelencephalon, in the medulla oblongata (MO).

Notice that there is a region on *Figure 3.7.C*, that is labelled as hindbrain (HB), since it has consistently GFP expression, but its anatomical identification and characterization is not assigned yet.

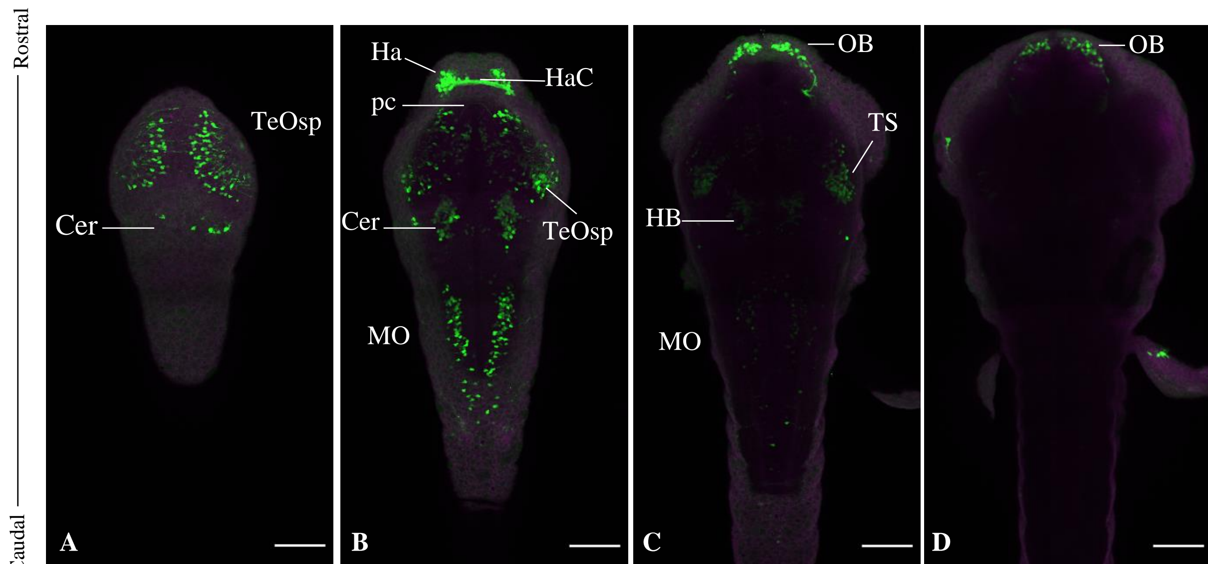


Figure 3.7 - **sst1: GFF Expression in the 3 dpf embryos.** A-D. z-projection of the 3 dpf brain of the sst1 line from dorsal (A) to ventral (D) after immunohistochemistry with anti-tERK (magenta) and anti-GFP (green) antibodies. The scale bar indicates 100 µm. Abbreviations: Cer –cerebellum, Ha –habenula, HaC – habenular commissure, HB – hindbrain, MO – medulla oblongata, OB – olfactory bulb, pc - posterior commissure, TeOsp – optic tectum stratum periventriculare, TS – torus semicircularis. Five samples were analyzed.

3.1.2.4. The sst1:GFF line at the 4, 5 and 6dpf stages

At these stages, in the telencephalon, it is possible to observe GFP expression in the pallium (P) and subpallium (subP) dorsally (*Figure 3.8.A-L*), and ventrally in the olfactory bulb (OB) (*Figure 3.8.C, G, K*).

In the diencephalon, there is GFP expressing cells in the habenula (Ha) and habenular commissure (HaC) (*Figure 3.8 .A-B, C-E, I-J*), and in the thalamus (Tha) (*Figure 3.8.C, H, L*).

In the optic tectum stratum periventriculare (TeOsp) (*Figure 3.8.B-C, E-G, I-J*) there is GFP expression. An in this same region – the mesencephalon – there is also in the torus semicircularis (TS) (*Figure 3.8.D, H, L*).

In the metencephalon, GFP expressing cells are present in the cerebellum (Cer) (*Figure 3.8.F-G, I-K*), and in the nucleus interpeduncularis (NIn) (*Figure 3.8.G-H, K-L*).

The medulla oblongata (MO) shows expression some cells expressing GFP, in the myelencephalon (*Figure 3.8.D-L*).

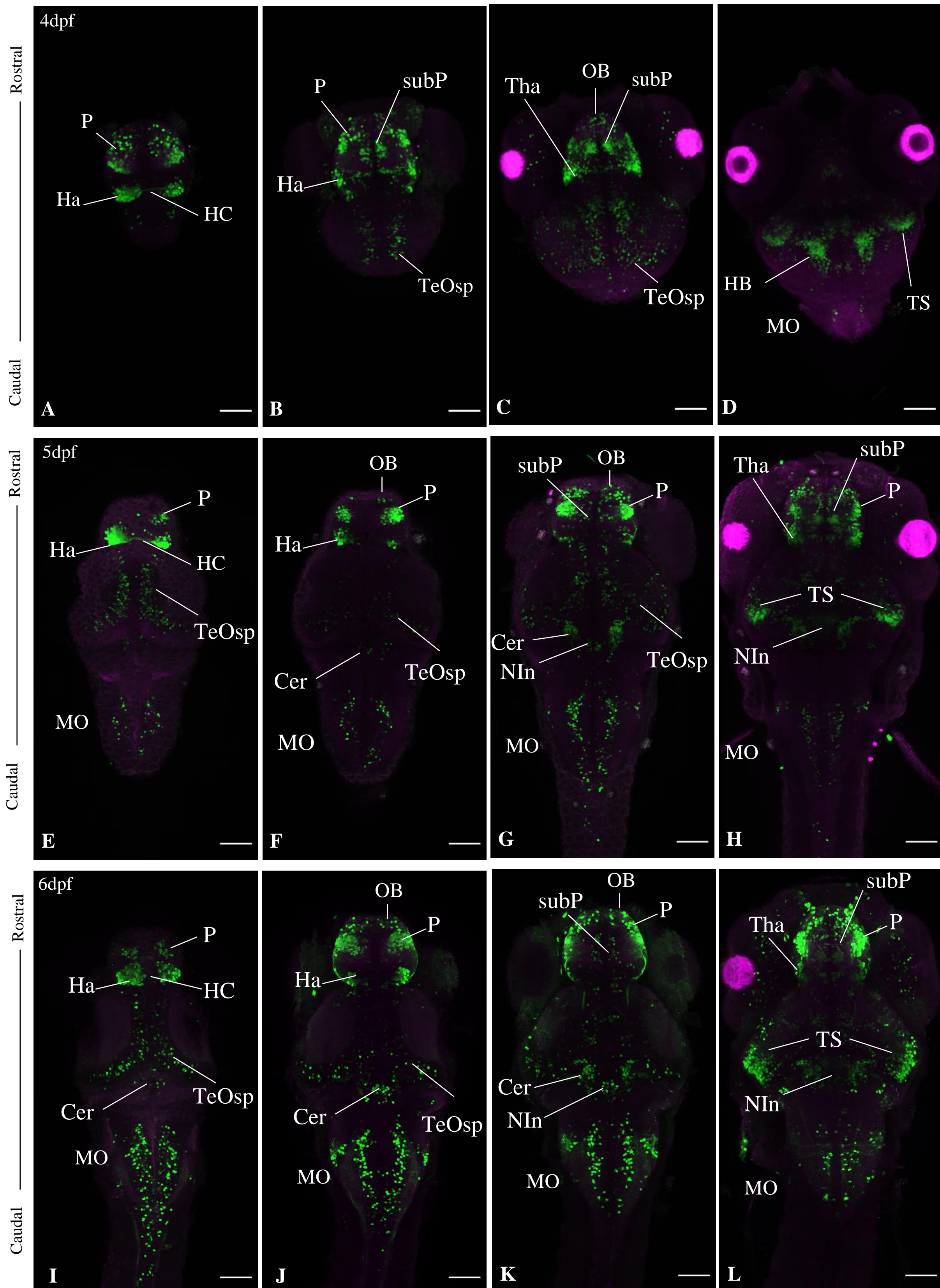


Figure 3.8 - **sst1: GFF Expression in the 4, 5 and 6 dpf embryos.** A-D, z-projection of the 4 dpf brain. E-H, z-projection of the 5 dpf brain. I-L, z-projection of the 6 dpf brain. All these images are from the sst1 line after immunohistochemistry with anti-tERK (magenta) and anti-GFP (green) antibodies. From dorsal (left) to ventral (right). The scale bar indicates 100 µm. Abbreviations: Cer –cerebellum, Ha –habenula, HaC – habenular commissure, HB – hindbrain, MO – medulla oblongata, NIn – interpeduncular nucleus, OB – olfactory bulb, P – pallium, subP – subpallium, pc - posterior commissure, TeOsp – optic tectum stratum periventriculare, Tha – thalamus, TS – torus semicircularis. Note: A-D shows a larva that is oriented in a slightly different way from E-L. In this way, on A-D there are structures more rostrally and dorsally located. Three samples were analyzed for the 4dpf stage, five samples for the 5dpf and four for the 6dpf.

3.1.2.5. sst1:GFF Overview

At 1dpf there is low levels of GFP expression. At 2dpf there is already some expression, mainly in the presumptive optic tectum (TeO), cerebellum (Cer) and medulla oblongata (MO). On this stage it is also possible to observe the formation of the stria medullaris (sm), in the telencephalon.

At the 3rd dpf there is GFP expression in the olfactory bulb (OB), in the habenula (Ha), and the habenular commissure (HaC), in the optic tectum stratum periventriculare (TeOsp), in the torus semicircularis (TS) and in the cerebellum (Cer). Even though this is not the establishment of the GFP expressing pattern, this pattern is going to be maintained until later stages. On this stage is also noticeable the expression in the posterior commissure (pc), that is not visible on later stages.

Besides the pattern described, at 4, 5 and 6 dpf, there is expression in the telencephalon, specifically in pallium (P) and subpallium (subP). The thalamus (Tha), in the diencephalon, is other structure that can only be seen on these stages, and also, in the metencephalon, the nucleus interpeduncularis (NIn).

3.1.2.6. sst1:GFF Expression Summary

In *Table 3.2* a summary of expression of the sst1:GFF line is presented.

Table 3.2 - **Overview of sst1 expression.** The regions listed above are following primarily the Z-brain atlas, but the ZBB and the Mueller et al, 2016 atlases were also consulted. (* - the structure listed can be assigned to other brain regions in the different atlases).

Brain Region	Time Range (dpf)	Areas with Expression	Abbreviations
Telencephalon	3-	Olfactory Bulb	OB
	4-6	Pallium	P
	4-6	Subpallium	subP
Diencephalon	3-6	Habenula	Ha
	3-6	Habenular Commissure	HaC
	4-6	Thalamus	Tha
	2	Stria Medullaris*	sm
	3	Posterior Commissure	pc
Mesencephalon	2	Optic Tectum	TeO
	3-6	Optic Tectum Stratum Periventriculare	TeOsp
	3-6	Torus Semicircularis	TS
Metencephalon	2-6	Cerebellum	Cer
	4-6	Nucleus Interpeduncularis	NIn
Myelencephalon	2-6	Medulla Oblongata	MO
Retina	No pattern observed		
Other	No pattern observed		

3.2. Discussion

3.2.1. Characterization of Pitx2c:GFF line

This transgenic line – pitx2c:GFF – is related with the pitx2 gene (paired-like homeodomain transcription factor 2), which is expressed during development in the brain of the zebrafish.

Although there is only a small expression of GFP in the otic placode on the 1st and 2nddpf, at the 3dpf stage there is expression throughout different regions of the brain. The basic pattern of expression of this line is, then, set on the 3rd dpf and at subsequent stages (4, 5 and 6dpf), there is an increase in cell number, but not a change in the observed pattern. The most prominent regions expressing GFP are the habenula and the nMLF. In the habenula, we observed an interesting asymmetry of the GFP expression with the left side of the habenula showing consistently more GFP expression than the right side. A similar asymmetry has been previously described for Pitx2c mRNA expression in 3dpf larvae, using *in situ* hybridization[26]. This asymmetry in Pitx2 mRNA expression is already present in 19 hpf embryos[27] but was only detected at 3 dpf in our line.

A line similar to the one used in our work has been previously characterized[28,30]. Comparing the line that we used here (GFF line), to the similar line previously described[28][30] (GFP line), there are some similarities but also relevant differences.

Both lines have expression in the nucleus of the MLF and oculomotor nucleus, although in the GFP line this is apparent at 1dpf while in our case it only appears at 3dpf [28]. Expression in the trigeminal ganglia is also observed in both lines.

In contrast, while the GFP line shows expression in the pallium, the subpallium, the posterior tuberculum, the hypothalamus and the superior raphe [30], none of those regions are expressing GFF in our line. Although the GFP line showed expression in the habenula, the asymmetry in the diencephalon observed in the GFF line was not found [30].

These differences in GFP expression might be due to the way these two lines were generated. Both are insertions of a construct that includes a similar *Pitx2c* genomic fragment driving either GFP or GFF, but they are independent insertions, most likely in a different region in genome and may thus be influenced by a different genomic landscape.

Overall, this line has GFP expression in fundamental parts of the brain that frame visually-guided behaviors[26–28] from the visualization of the stimulus to the encoding of a proper response. Therefore, it may be useful to study the development of those regions.

3.2.2. Characterization of sst1:GFF line

The *sst1* gene is associated with regulation of locomotor functions[29]. This fact might be valuable to understand how the neuronal circuits associated to visually guided behaviors develop.

The GFP expression pattern on this line is visible after 1dpf: at 2dpf there are well defined regions and then, after 4dpf the expression pattern is established. On later stages (5 and 6dpf) the expression pattern is maintained, showing only an increase in cell number.

The expression in the cerebellum matches the expression previously found on 5dpf larvae through *in situ* hybridization[29]. However, none of the other expressing cell clusters described by the same article – preoptic area and hypothalamus – can be found here. Moreover, the preotic area, hypothalamus and pituitary were also described on 3dpf larvae using *in situ* hybridization[34], and on 7dpf fish using a transgenic line[35]. We could not verify these regions since these structures are outside of our z-stack range – deeper inside the fish.

The stria medullaris, that here can only be seen on 2 dpf larvae, it's an afferent connection to the habenula[43]. This is the path that allows inputs from the forebrain to connect to the habenula. Even though, this structure is not present in later stages, on those there is the habenular commissure, that connects the right habenula to the left habenula[43].

This line has GFP expressing cells in brain regions that are essential for visually guided behaviors, from receiving the stimulus, interpreting it and sending a motor response. Some of these are visible through GFP expression since early stages, like the olfactory bulb (OB), habenula (Ha), optic tectum (TeO), cerebellum (Cer), torus semicircularis (TS) and medulla oblongata (MO). Others are only expressed later in development like the pallium (P), subpallium (subP), thalamus (Tha) and nucleus interpeduncularis (NIn).

4. Establishing a Reference Marker and Sample Orientation for the Generation of a Brain Atlas for the 2dpf Zebrafish Embryo

4.1. Results

4.1.1. Staining Comparison

Currently, our long-term goal is to create brain atlases through development of the transgenic lines generated at the Orger Lab. This requires the use of stains that make the whole brain visible allowing the identification of the different brain region. The method most used so far for zebrafish larval brain is the detection of the protein ERK (extra cellular response kinase).

The protein ERK is a kinase involved in signaling cascades in several processes in development and homeostasis and is also associated to neuronal activity. Phosphorylation of ERK has been used as a maker of neuronal activity[31]. As ERK is expressed in most neurons, detection of this protein with an antibody known as anti-tERK or total ERK (detects both the phosphorylated and no-phosphorylated forms) has been used as counter staining for GFP expression in atlases of the 6dpf larvae brain[31,32].

To do this, suitable counter staining alternatives to anti-tERK antibody should be explored for the reference stage (6dpf) but also for early stages (2dpf). These alternatives should grant an overview of the whole brain and should be able to undergo successfully through image registration.

To investigate the possibility of alternatives with potential advantages (e.g. better tissue penetration, live imaging, applicability to embryonic and earlier larval stages), here we examine, not only the anti-tERK antibody, but also a transgenic line with pan neuronal fluorescence (alphaTubulin:mScarlet)[38] and two dyes: the DiD[39] and the BODIPY TR methyl ester[40]. For this study we used 6dpf larvae – to compare with the previously described atlases – and also 2dpf embryos – to evaluate if the counter staining alternatives can be advantageous in early stages.

Please note that all comparisons are qualitative.

Table 4.1 - **Number of samples per treatment and stage.** Number of samples used and analyzed per treatment and stage.

Treatment	Stage	Number of Samples
tERK staining	2dpf	7
	6dpf	6
mScarlet	2dpf	5
	6dpf	4
DiD	2dpf	3
	6dpf	6
BODYPI TR methyl ester	2dpf	3
	6pdf	6

4.1.1.1. mScarlet

mScarlet[44] is a monomeric fluorescent protein that can be used as reporter of gene expression. This is the brightest mRFP available at the moment and does not present cytotoxicity being a good staining for living cells[44]. Here, a transgenic line – alphaTubulin:mScarlet – was used. This line was generated by cloning the *mScarlet* encoding gene under the control of the alpha-1-Tubulin promotor[38]. In this way, mScarlet has pan neuronal expression inside cells. We explored the use of this fluorescent protein *in vivo*, following what was already described[44] but also in fixed tissue, assessing how it works after fixation and immunohistochemistry.

Figure 4.1, is an example of live imaging of a 2dpf embryo and it is possible to observe the bright expression through the whole brain. With fixed samples, mScarlet is visible at early stages, but slightly less bright than the live image (*Figure 4.2 and 4.3*). At 6dpf, mScarlet expression is very bright and well distributed through the brain. There are some conspicuous brain regions that can be easily identified such as the olfactory bulb (OB) and the habenula (Ha).

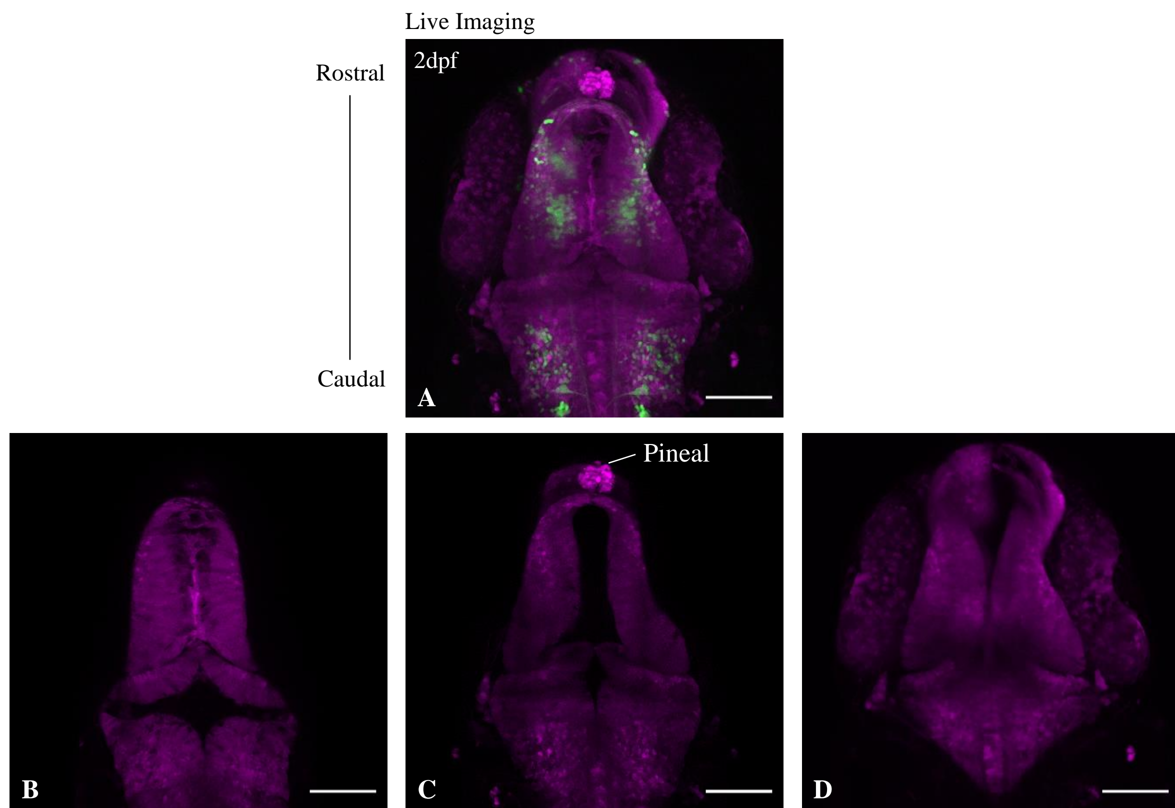


Figure 4.1 - **mScarlet Expression in 2dpf Living Embryo.** A, Maximum projection of the 2 dpf brain of the lx200:GFF;UAS:GFP; alphatubulin-mScarlet larva after immunohistochemistry with anti-GFP (green) antibody. mScarlet can be found in magenta. B-D, z-projections from dorsal (B) to ventral (D), showing only the mScarlet channel (magenta). The scale bar indicates 100 µm.

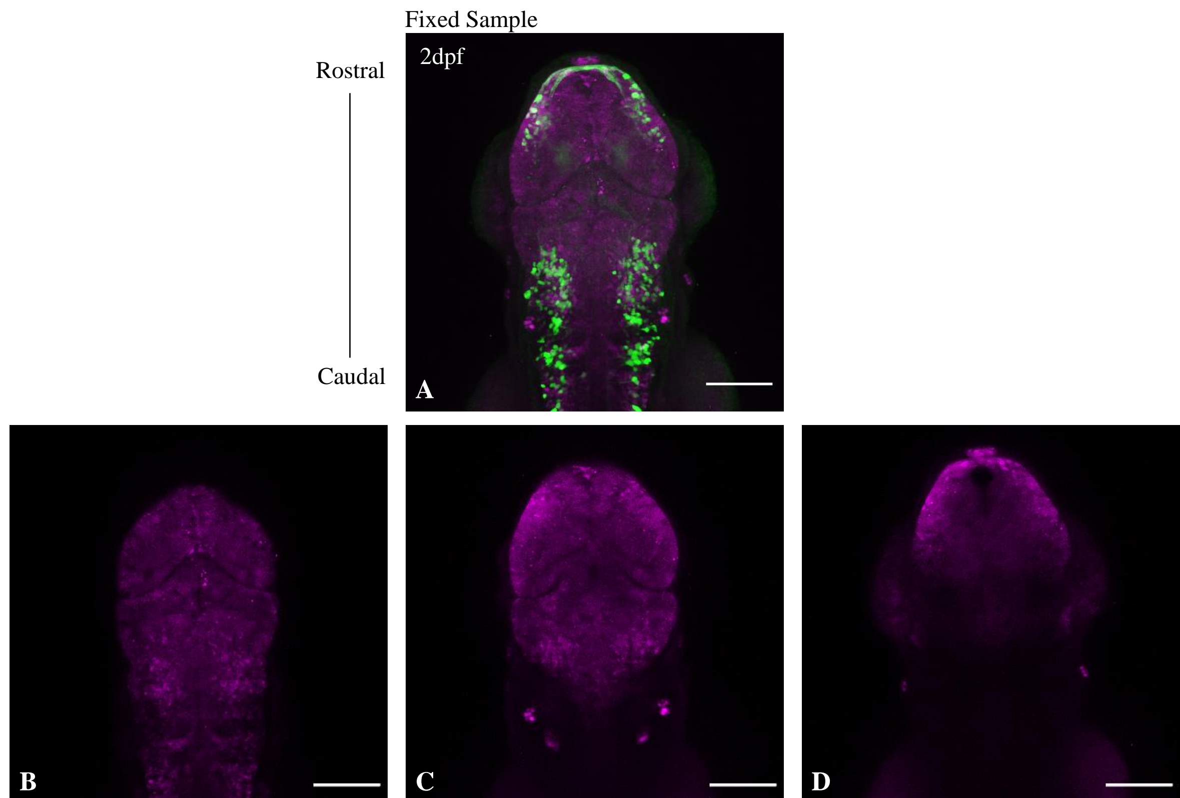


Figure 4.2 - **mScarlet Expression in 2dpf Fixed Embryo.** A, Maximum projection of the 2dpf brain of the lx200:GFF;UAS:GFP ; alphatubulin-mScarlet line after immunohistochemistry with anti-GFP (green) antibody. mScarlet can be found in magenta. B-D, z-projections from dorsal (B) to ventral (D), showing only the mScarlet channel (magenta). The scale bar indicates 100 µm.

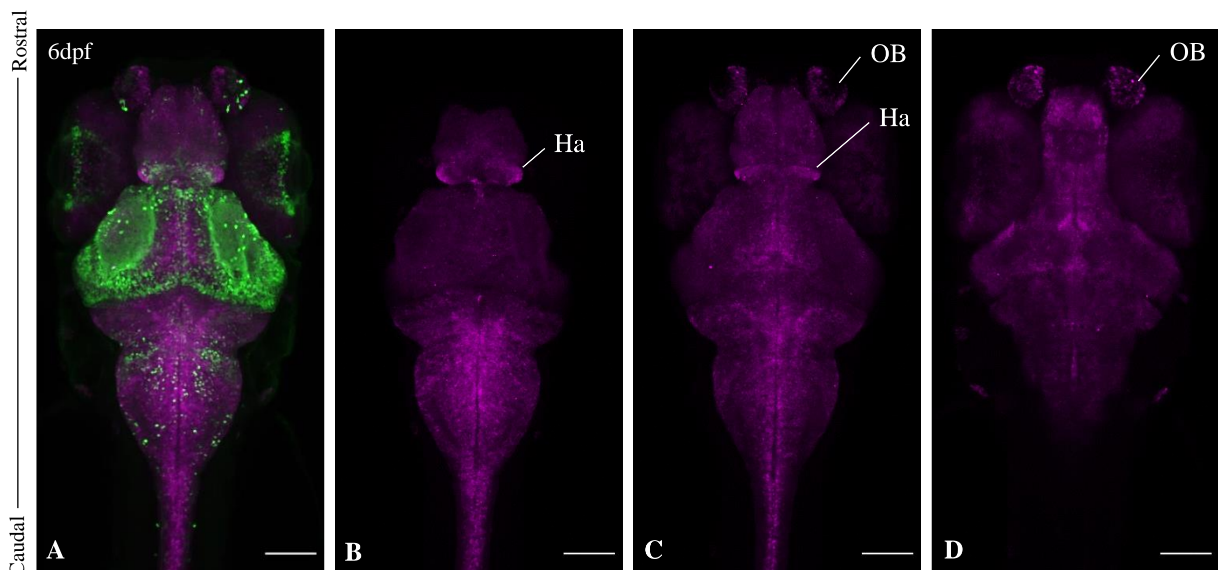


Figure 4.3 - **mScarlet Expression in 6dpf Fixed larvae.** A, Maximum projection of the 6dpf brain of the lx200:GFF;UAS:GFP; alphatubulin-mScarlet line after immunohistochemistry with anti-GFP (green) antibody. mScarlet can be found in magenta. B-D, z-projections from dorsal (B) to ventral (D), showing only the mScarlet channel (magenta). The scale bar indicates 100 µm.

4.1.1.2. DiD

DiD is a far-red fluorescent dye. Its lipophilic property allows DiD to stain cell membranes and lipidic vesicles.

DiD has, overall, a smooth staining throughout the whole brain, at any stage. In early stages (2dpf), DiD stains the yolk due to its lipophilic property, over-staining it (*Figure 4.4*). This creates some attenuation in the brain. It is also possible to observe that mostly at 2dpf there are some residues around the head and yolk.

At later stages, like the 6 dpf (*Figure 4.5*) DiD stains the whole brain, allowing the visualization of the pineal gland, the optic tectum neuropil (TeOn), the pallium and subpallium (P and subP), and the lenses. As can be seen on *Figure 4.5.C-D*, DiD has good penetration of the tissues, since both the outer and the deeper layers of the brain have similar levels of brightness. It's also possible to observe blood vessels throughout the brain.

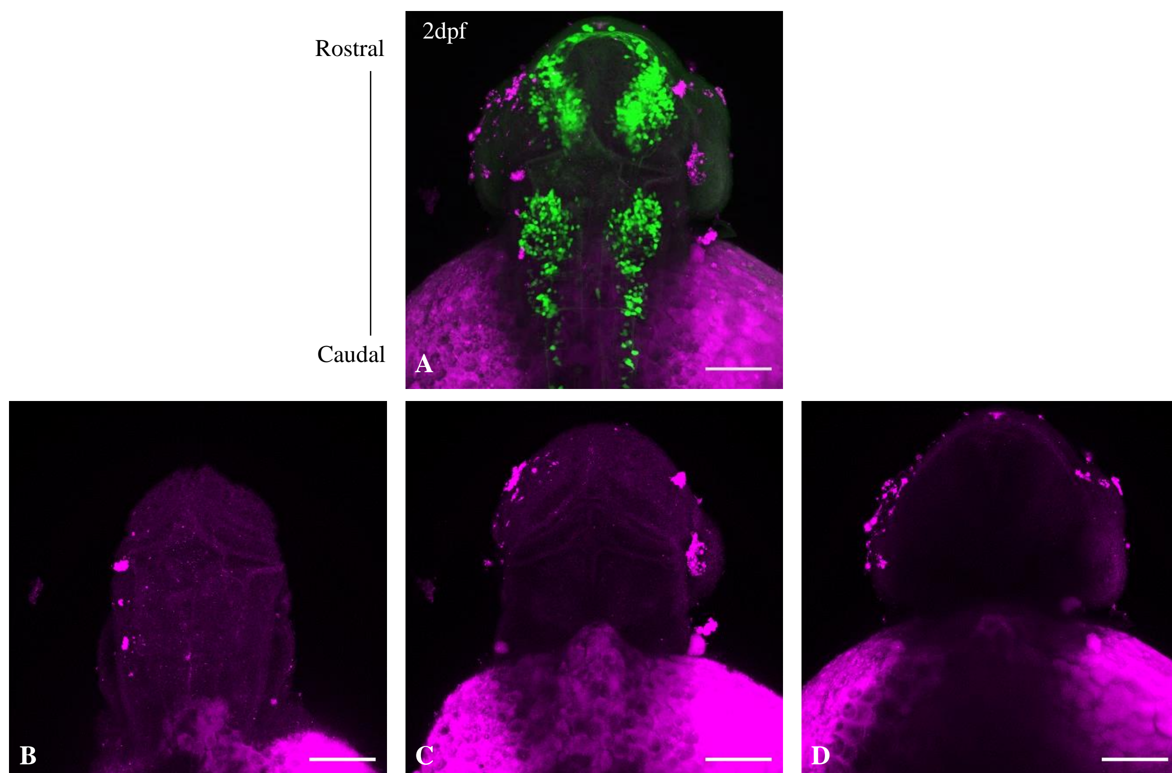


Figure 4.2 - **DiD Expression in 2dpf.** A, Maximum projection of the 2dpf brain of the lx200:GFF;UAS:GFP line after immunohistochemistry with anti-GFP (green) antibody and staining with DiD (magenta). B-D, z-projections from dorsal (B) to ventral (D), showing only the DiD channel (magenta). The scale bar indicates 100 µm.

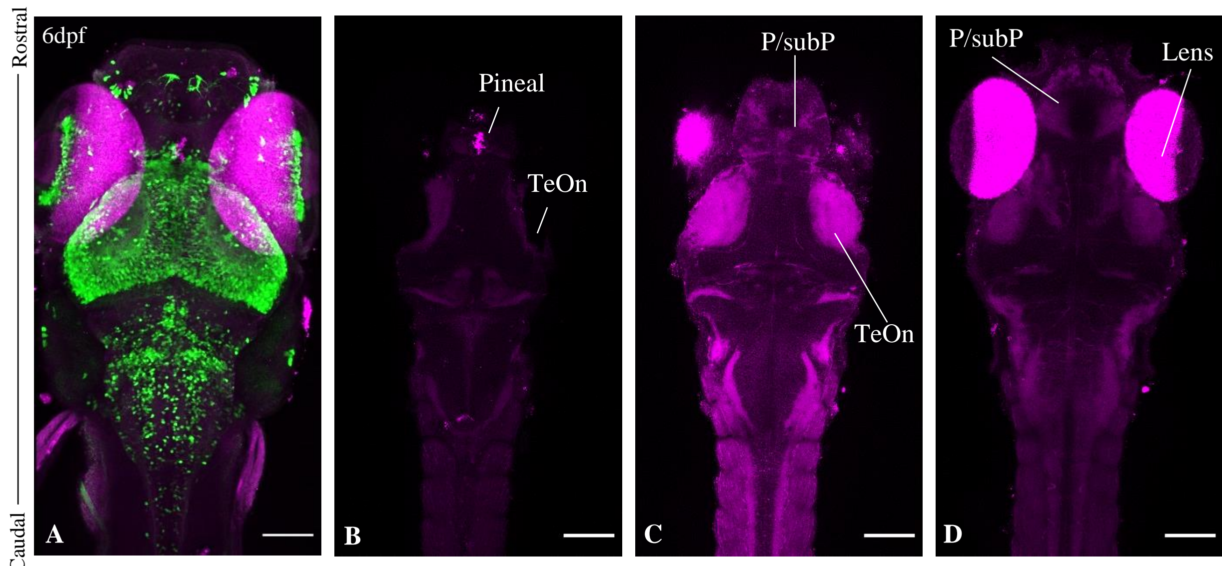


Figure 4.3 - **DiD Expression in 6dpf.** A, Maximum projection of the 6dpf brain of the lx200:GFF;UAS:GFP line after immunohistochemistry with anti-GFP (green) antibody and staining with DiD (magenta). B-D, z-projections from dorsal (B) to ventral (D), showing only the DiD channel (magenta). The scale bar indicates 100 µm.

4.1.1.3. BODIPY TR methyl ester

BODIPY TR methyl ester dye is a synthetic red fluorophore that can enter the cells and that stains endomembranous organelles[40].

BODIPY TR methyl ester dye is known to be a suitable contrast alternative for GFP expression[40]. This dye is usually applied *in vivo* and can be used for live imaging or fixed tissue.

As seen in *Figure 4.4*, BODIPY TR does not have a good penetration of the tissue since early stages, showing always more fluorescence on the surface than on the inside of the brain. Especially, at 6 dpf (*Figure 4.5.A-D*), staining on the skin cells is so bright that, the inside of the fish can't be seen through a maximum projection.

Alternatively, we have also used BODIPY TR following fixation of the samples. For this, we performed an immunohistochemistry protocol and then stained them with BODIPY TR for 5 days. In this way, BODIPY TR is not so visible on the surface, and a pattern can be seen on the brain, particularly in the optic tectum neuropil (*Figure 4.5.E-H*).

In both protocols used for BODIPY TR methyl ester, blood vessels seem to be stained by this dye.

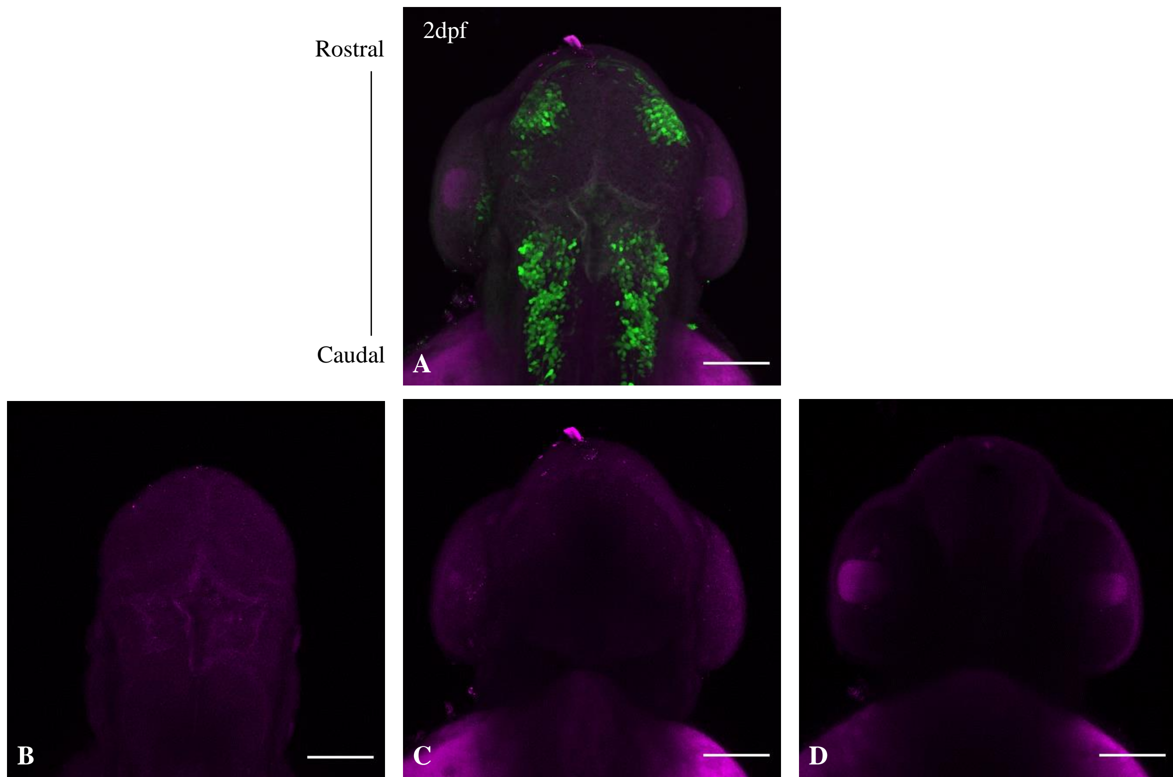
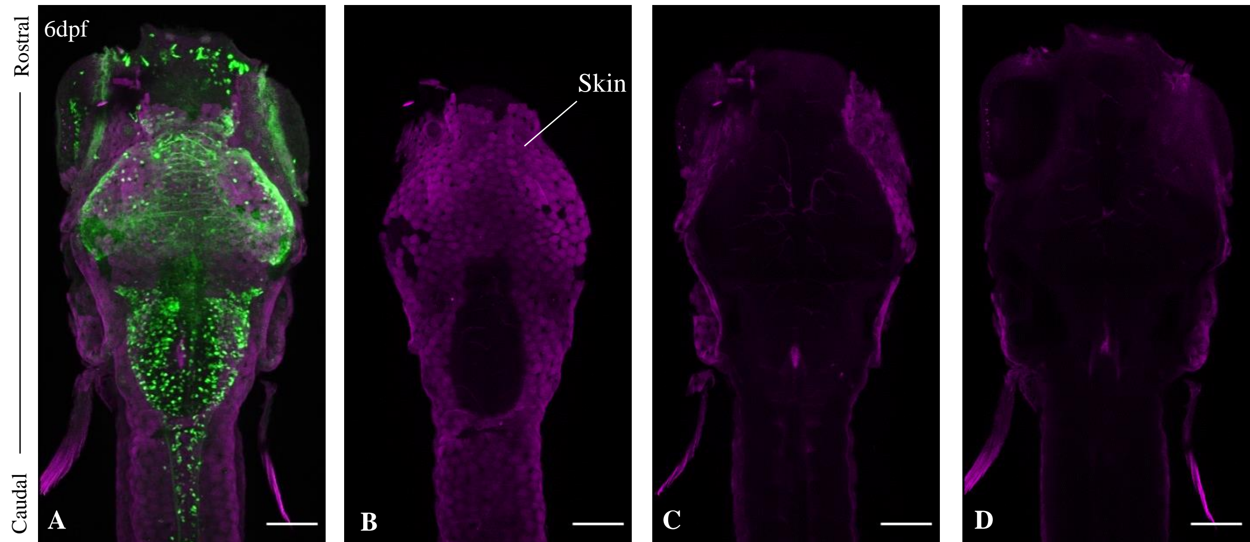


Figure 4.4 - **BODIPY TR Expression in 2dpf Fixed Embryo.** A, Maximum projection of the 2dpf brain of the lx200:GFF;UAS:GFP line after immunohistochemistry with anti-GFP (green) antibody. BODIPY TR can be found in magenta. B-D, z-projections from dorsal (B) to ventral (D), showing only the BODIPY TR channel (magenta). The scale bar indicates 100 µm.



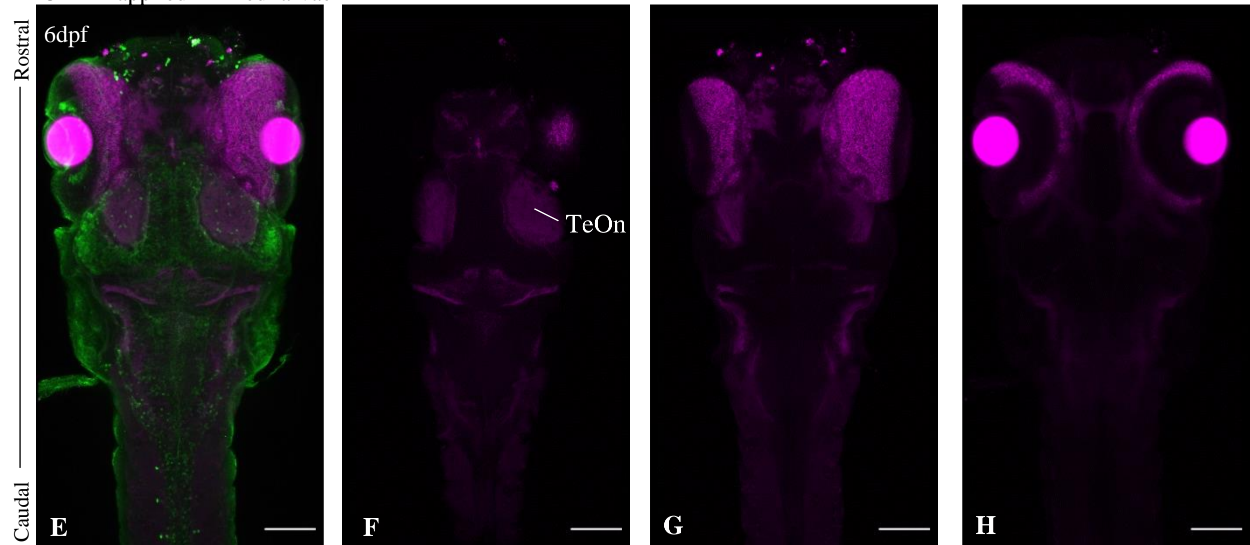


Figure 4.5 - **BODIPY TR Expression in 6dpf.** A, E, Maximum projection of the 6dpf brain of the lx200:GFF;UAS:GFP line after immunohistochemistry with anti-GFP (green) antibody and staining with BODIPY TR (magenta). B-D, F-H, z-projections from dorsal (left) to ventral (right), showing only the BODIPY TR channel (magenta). From A-D, BODIPY TR was applied as described by Cooper et al., 2005, on their protocol. From E-H, BODIPY TR was applied after fixing and immunohistochemistry. The scale bar indicates 100 µm.

4.1.1.4. Anti-tERK

The anti-tERK antibody recognizes extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), which are expressed in most neurons. In this way, staining with this antibody allows the visualization of the whole brain of the fish.

anti-tERK antibody staining (tERK staining) is apparent since early stages, as seen in *Figure 4.8*. At 2dpf, compared with 6dpf, tERK staining seems to be dimmer. At 6dpf, there are structures that express tERK in higher levels than others. Examples of this structures are the optic tectum neuropil (TeOn), pallium and subpallium (P and subP) and the lenses (*Figure 4.9*).

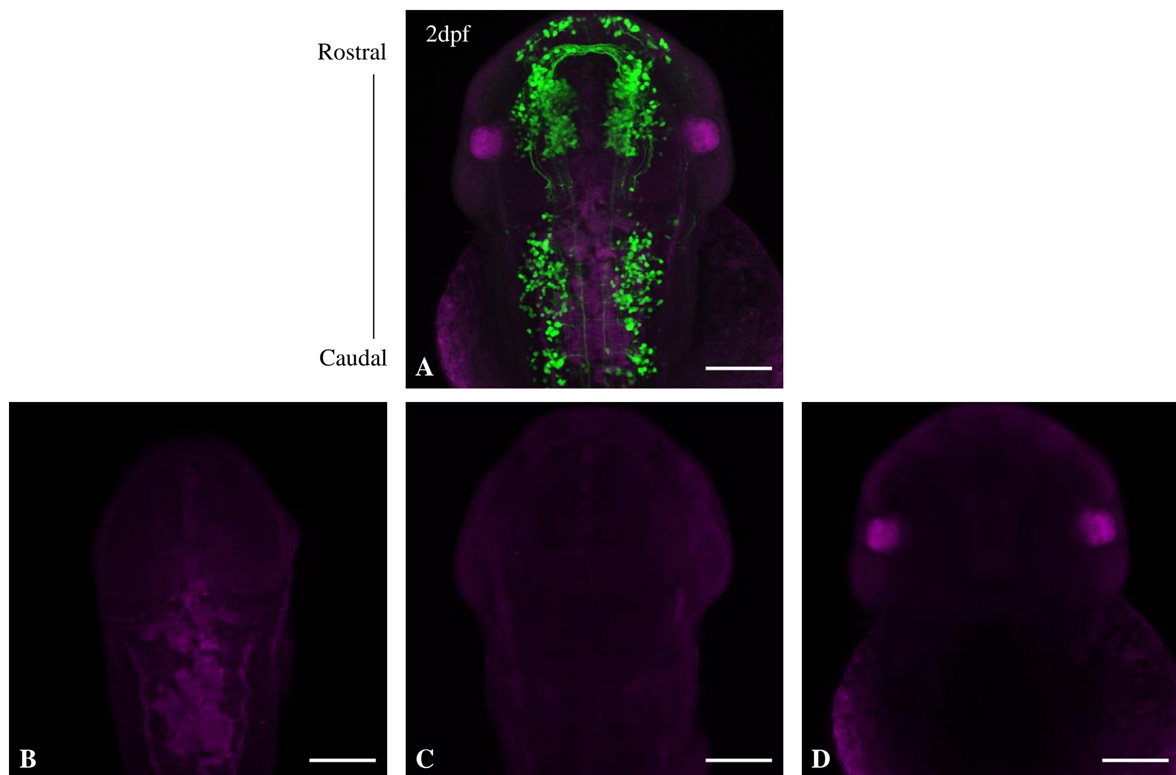


Figure 4.6 - **tERK Expression in 2dpf.** A, Maximum projection of the 2dpf brain of the lx200:GFF;UAS:GFP line after immunohistochemistry with anti-tERK (magenta) and anti-GFP (green) antibodies. B-D, z-projections from dorsal (B) to ventral (D), showing only the anti-tERK channel (magenta). The scale bar indicates 100 µm.

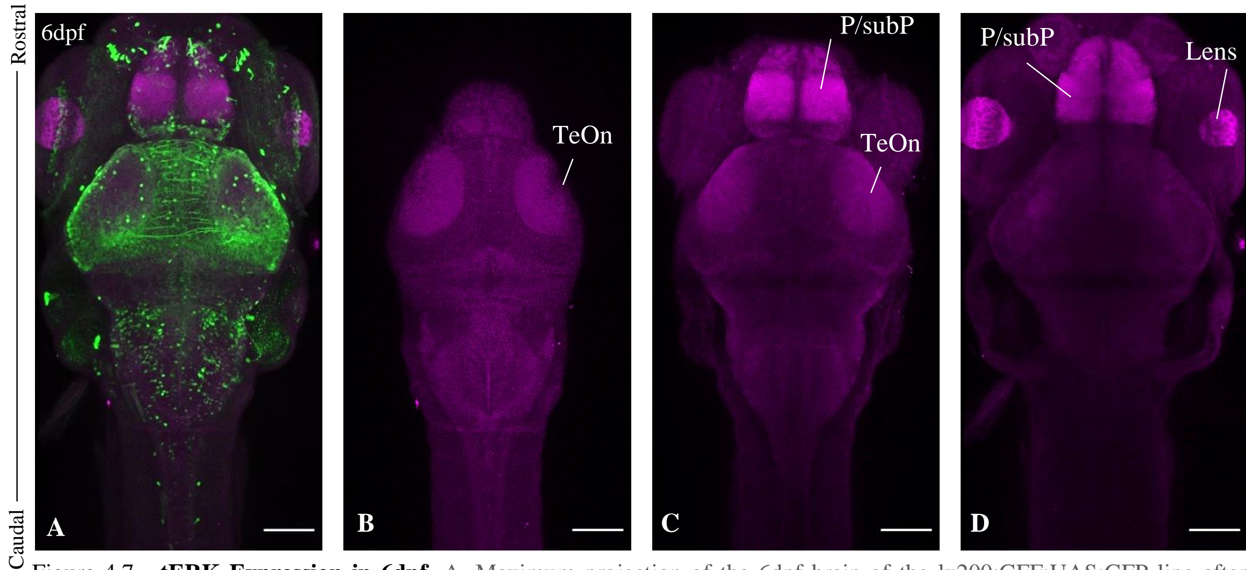


Figure 4.7 - **tERK Expression in 6dpf.** A, Maximum projection of the 6dpf brain of the lx200:GFF;UAS:GFP line after immunohistochemistry with anti-tERK (magenta) and anti-GFP (green) antibodies. B-D, z-projections from dorsal (B) to ventral (D), showing only the anti-tERK channel (magenta). The scale bar indicates 100 µm.

4.1.5. Overview and comparison of general staining methods used

At 2dpf live imaging of mScarlet is the best option in terms of brightness and evenness through the brain.

For fixed embryos at 2dpf, mScarlet is also the best option since it has brain regions more defined and has slightly more brightness, comparing to the other types of staining. tERK, DiD and BODIPY TR methyl ester qualitatively have lower levels of brightness compared with mScarlet, for this stage.

Regarding the capacity of tissue penetration, *Figure 4.10* summarizes the qualities and defects of each type of staining at 6dpf. In this set of images, it is possible to qualitatively compare staining since it presents approximately the same brain sections. It is possible to see, that in the deepest regions of the brain there is an attenuation of the expression of all stainings (*Figure 4.10.*A_1-E_1). tERK staining is the brightest staining, showing evenly expression through the brain. Even though not so bright, mScarlet and DiD have fixed patterns of expression, which would be helpful for the registration process. The BODYPI TR is the one that have less expression inside the brain, showing some blood vessels brighter than brain regions. With the method of applying BODYPI TR after fixation and immunohistochemistry, the pattern is more distributed and brighter, but still not comparable to tERK staining, mScarlet and DiD.

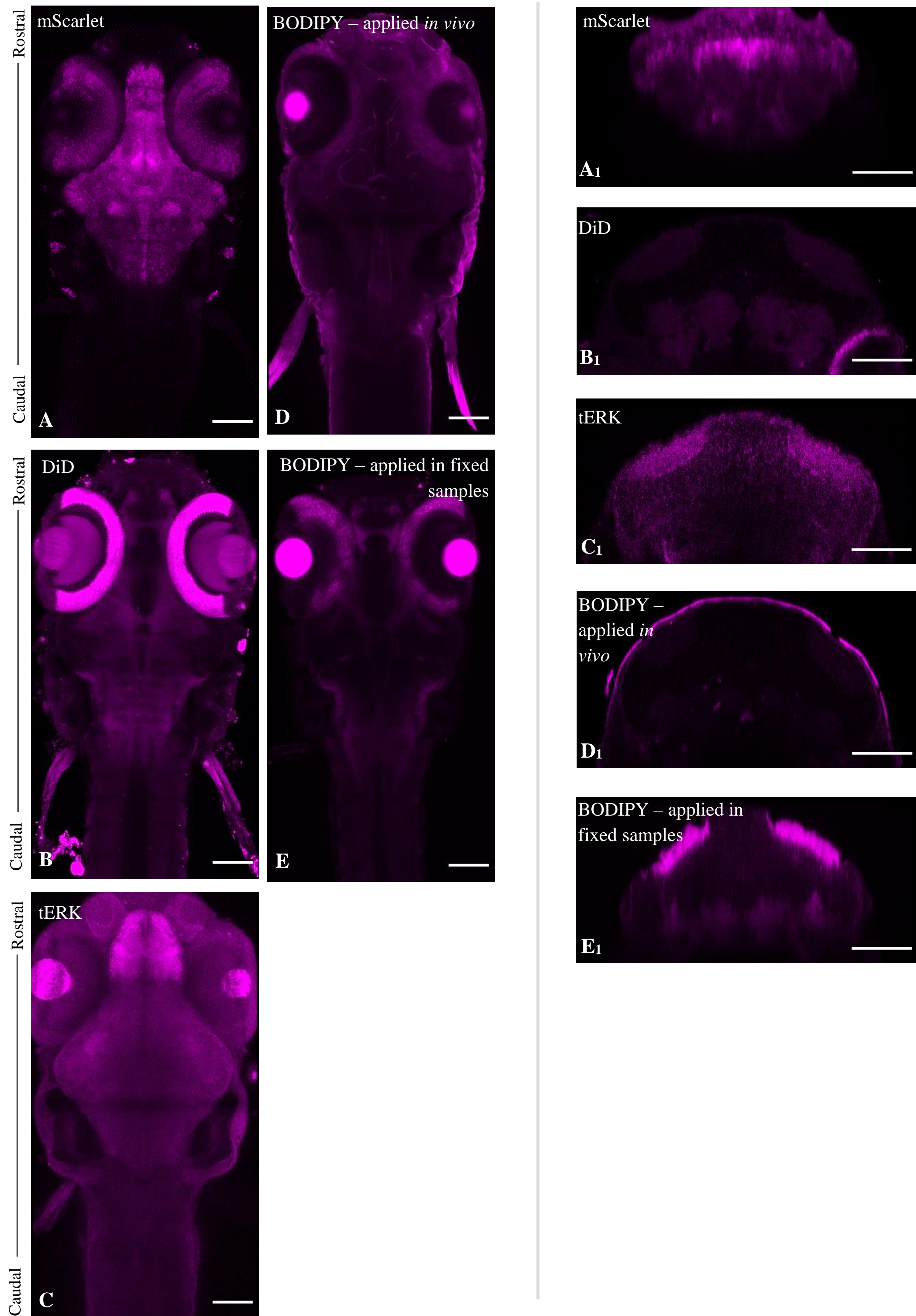


Figure 4.8 - **Expression of the various staining alternatives on deeper slices.** Maximum projection of deeper slices in the brain of the 6 dpf larvae with mScarlet expression (A), DiD expression (B), tERK expression (C) and BODIPY expression (D and E). On the right-side panel there is an orthogonal view slice of the brain for each staining: mScarlet expression (A_1), DiD expression (B_1), tERK expression (C_1) and BODIPY expression (D_1 and E_1). The scale bar indicates 100 µm.

4.1.2. Mounting Position Comparison

Currently, an atlas for the early stages of brain development is not available. In this way and being a long-term goal of ours to create a comprehensive brain atlas through development, we faced a challenge while positioning embryos to image. Embryos at the 1dpf and 2dpf stage, present a curvature between the forebrain and midbrain that makes imaging of the whole brain difficult to achieve.

Here we address this issue by comparing two main different positions of mounting (Horizontal – conventional – and Vertical – alternative (*Figure 2.1*). We also tried to use these positions with a Confocal microscope and with a Light Sheet microscope, since the first one has higher resolution and the second one allows the rotation of the samples.

We used a combination of anti-GFP and anti-tERK for the confocal images and a combination of anti-GFP and mScarlet for the light sheet images.

4.1.2.1. Horizontal Position – Conventional

Horizontal position (*Figure 4.11*), is the conventional way of mounting zebrafish larvae in later stages, laying the larvae with their back in contact with the coverslip. In this way, part of the forebrain, the midbrain and part of the hindbrain are shown.

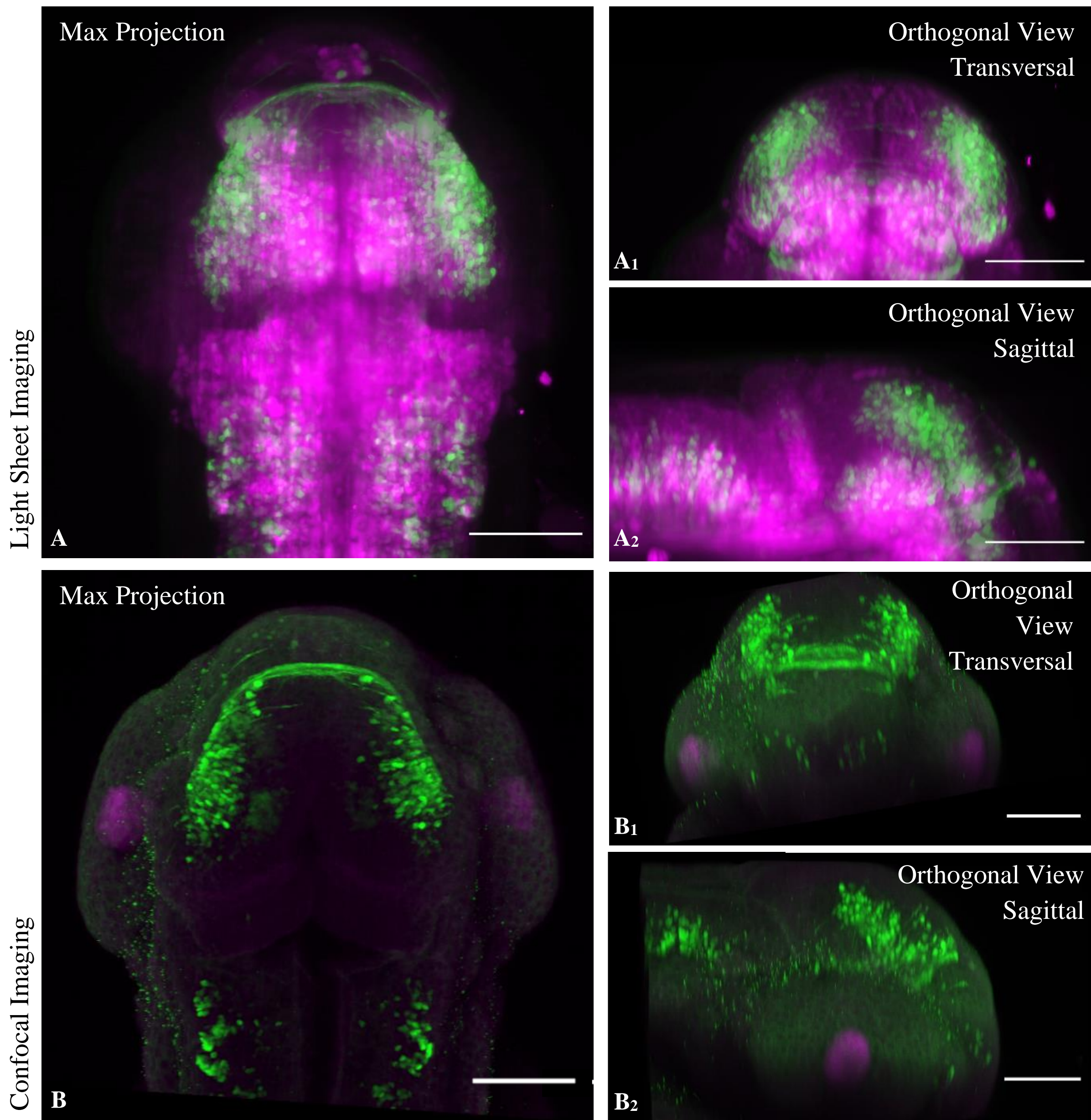


Figure 4.9 - **Horizontal Position – Conventional.** A. Light Sheet image of Horizontal Position. Maximum projection of the 2 dpf brain of the lx200:GFF line and alphaTubulin:mScarlet line after immunohistochemistry: GFP (green) and mScarlet (magenta). A1-2. Orthogonal views of that z-stack. B. Confocal Image of Horizontal Position. Maximum projection of the 2dpf brain of the lx200:GFF line after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). $B_{1\text{-}2}$. Orthogonal views of that z-stack. The scale bar indicates 100 µm.

4.1.2.2. Vertical Position – Alternative

Vertical position (*Figure 4.12*), is an alternative method of mounting embryos in the early stages, getting the rostral part of the brain being in contact with the coverslip. With this position, the forebrain becomes more available to image. The midbrain can be completely observed, while the hindbrain is less acessible to image.

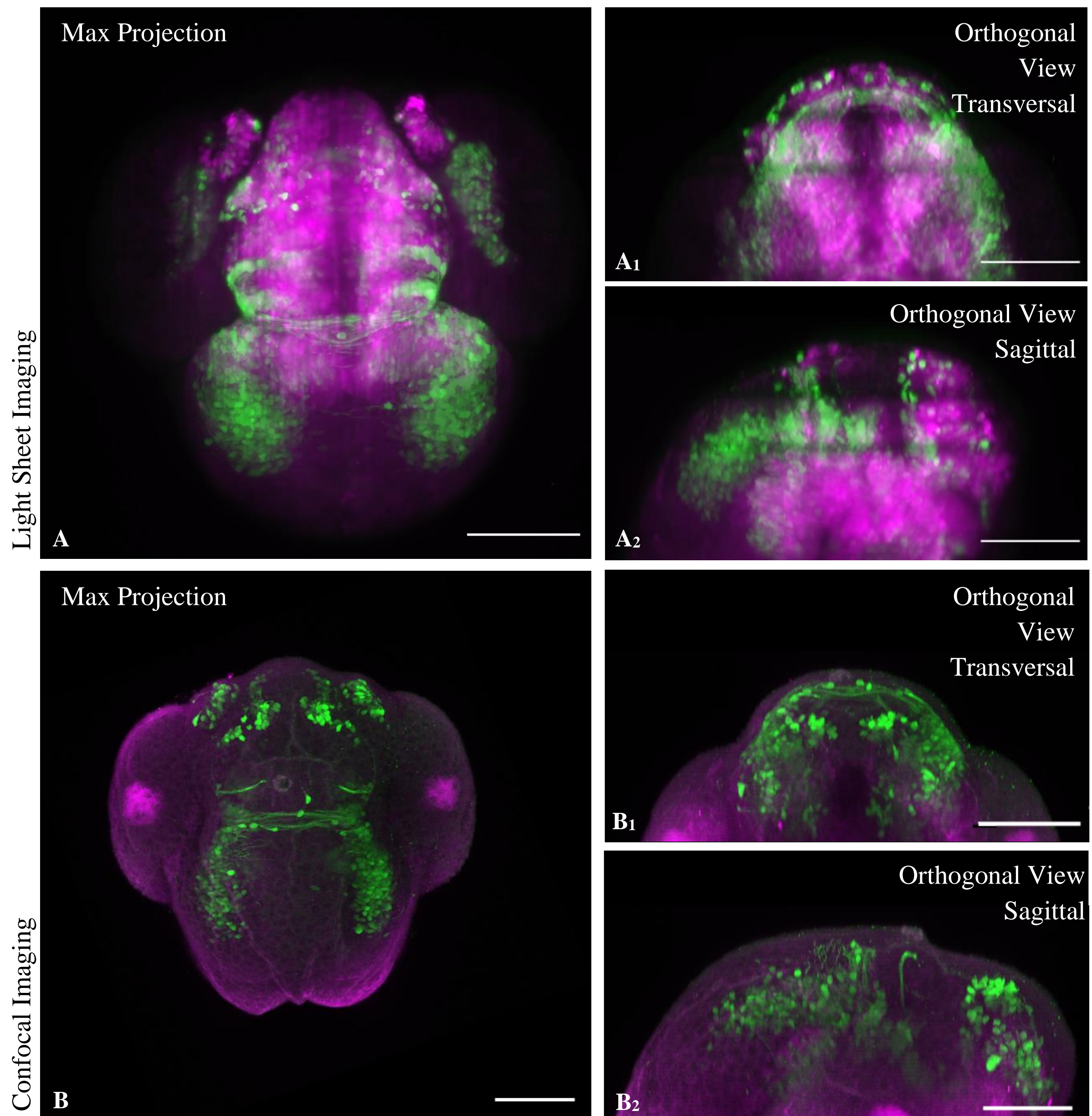


Figure 4.10 - **Vertical Position – Alternative.** A. Light Sheet image of Vertical Position. Maximum projection of the 2 dpf brain of the lx200:GFF line and alphaTubulin:mScarlet line after immunohistochemistry: GFP (green) and mScarlet (magenta). A1-2. Orthogonal views of that z-stack. B. Confocal Image of Vertical Position. Maximum projection of the 2dpf brain of the lx200:GFF line after immunohistochemistry with anti-GFP (green) and anti-tERK (magenta). B_{1-2}. Orthogonal views of that z-stack. The scale bar indicates 100 µm.

4.1.2.3. Other Positions – Alternative

These positions can be achieved by rotation of the sample through the Light Sheet microscope, showing a more dorsal view (*Figure 4.13.A*) to more rostral and ventral views (*Figure 4.13.B-C*), depending on the structures chosen to be studied.

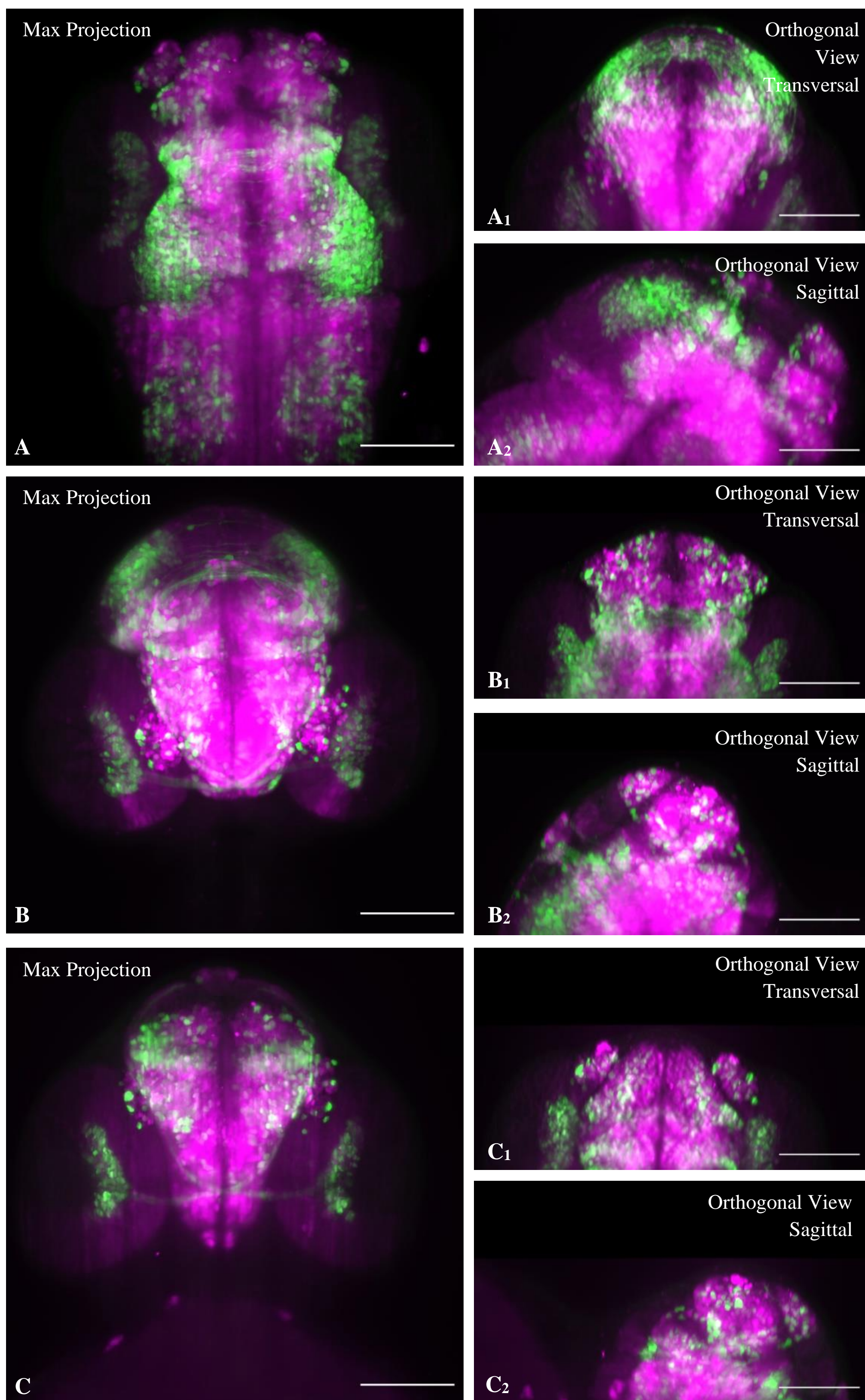


Figure 4.11 - **Alternative positions with Light Sheet Imaging.** A-C. z-stacks showing from dorsal (A) to rostral (B) alternatives positions for imaging. Maximum projection of the 2 dpf brain of the lx200:GFF line and alphaTubulin:mScarlet line after immunohistochemistry: GFP (green) and mScarlet (magenta). A-C_{1-2}. Orthogonal views of each z-stack. The scale bar indicates 100 µm.

4.1.2.4. Mounting Positions Overview

When comparing horizontal and vertical position it is possible to see that the different views allow a better visualization of different structures in space. With the vertical position, GFP expressing cells are more visible in the forebrain and commissures are more defined. With the horizontal position there is more information available on the hindbrain, but on the forebrain, cells seem to be overlapping, being difficult to distinguish the brain regions in which they are on. In this way, the Vertical position can contribute to enrich the information that we are gathering about these transgenic lines.

The main advantage of using the Light Sheet microscope is that the same sample can be repositioned many times in one session. In fact, the images of Light Sheet microscopy gathered here are from the same fish, and with that in mind, analyzing and annotating specific brain regions can be simplified by reposition the embryo until having the GFP expressing cells, on that region, visible. The same positions as the confocal mounting can be achieved (*Figure 4.11 and 4.12*) and alternatives to these positions can also be generated from more dorsal views (*Figure 4.13.A*) to more rostral views (*Figure 4.13.C*). Comparing this type of microscopy with confocal microscopy, the latter better resolution making possible to image and annotate single cells and axons, which are more reliable for registration.

4.2. Discussion

4.2.1. Staining Comparison

tERK antibody is now widely used as a reference counter staining since it's present in the most used zebrafish brain atlases[31,37]. This is a pan-neuronal antibody, which expression is present since early stages of development. In the beginning of this project, one of the main problems that we found when using tERK staining, was that the tissue penetration was not good enough, having a dimmer expression inside of the brain. This was an issue that occurred both in early and later stages. As consequence, tERK antibody alternatives for GFP counter staining were explored.

The alphaTubulin:mScarlet transgenic zebrafish line was recently generated in the Michael Orger lab[38] as a counter staining for live imaging. Here we examined this line's expression in living and fixed embryos and larvae. mScarlet in live imaging has a very bright expression through the whole brain and without any tissue penetration problem in living fish. Notably, in fixed samples, this expression is still very bright, but not as bright as in living fish. This difference in expression might be due to factors that diminish the mScarlet expression, such as the fixation with paraformaldehyde and the bleaching from the exposure to the laser while acquiring images. Either way, brain regions have defined boundaries, which is required for a good registration of images into the atlases.

Other counter staining that we explored was the DiD[39]. This is a lipophilic dye that stains cell membranes and lipidic vesicles. Although, in early stages, the yolk tends to be brighter due to its amount of lipids (shadowing the brain), on later stages DiD seems to have a better tissue penetration. On 6dpf, there are well defined structures in the brain that can be used to the registration process. A problem that DiD has is the formation of residues and crystals around the sample. These residues are usually very bright and may be a challenge for registration since the algorithm might take it as part of the brain. This might be easily solved introducing a step of extra washes after applying this dye. Other challenge that might rise in registration is the staining of blood vessels: DiD seems to have some affinity with blood vessels, and we have noticed that these blood vessels differ on each individual.

The last dye that we tested was the BODIPY TR methyl ester[40]. This dye was designed to be used in living samples, but here we investigated its use in fixed tissue. We used two different protocols: applying the dye *in vivo* and fixing the samples after; and fixing the samples and applying the dye after. The first protocol was already described[40]. We noticed that applying BODIPY TR *in vivo* makes the surface of the fish look brighter than the inside of the brain, showing a bad penetration of the tissue. With the second method – fixing the samples and applying the dye – the expression is more even between the surface and the inside, but still showing a very dimmer expression. Nevertheless, a pattern can be seen through the whole brain.

At early stages, all types of stainings show small brightness through the whole brain: either in surface or deeper inside the brain. mScarlet is the only kind of staining that shows more defined brain regions, and qualitatively more brightness and better tissue penetration than the others. Its brightness is even more visible in living embryos. We believe that this – mScarlet – might be a potential alternative for tERK staining in early stages and, more tests from 1 to 3 dpf should be done to guarantee the quality of this line.

tERK staining seems to be the brightest even in deeper slices of 6dpf fish. Qualitatively DiD and mScarlet seem to be the best alternatives to tERK staining. DiD shows a good tissue penetration, that can even be improved by extending the staining time. At the same time, mScarlet, being constitutively expressed in the cells, is shown throughout the tissue, even deeper inside the brain. Adding to this, its versatility of use in living and fixed samples is a great advantage. These two alternatives still have some problems that need to be further explored – DiD has shown some debris on the surface of the samples and it may not have consistency when applied for registration and mScarlet shows less brightness in fixed samples than in living samples.

BODIPY TR methyl ester treated samples, seems to display weaker signal than the other alternatives, and lack in a defined pattern which may preclude its use for image registration. Even though this was a good alternative to live imaging, alphaTubulin:mScarlet line might be more adequate for this kind of technic.

At the moment, we are still using tERK staining as our counter staining considering that we improved the protocol to have a better tissue penetration. This is also the most used counter staining in zebrafish atlases, making our results more comparable.

Nevertheless, DiD and mScarlet seem to have potential advantages, and should be examined in the future. On one hand, DiD should be submitted to image registration to see if it can be consistently used as an alternative counter staining. On the other hand, mScarlet seems to have great potential for live imaging, which should be tested for more developmental stages and seems to be a great alternative for staining early stages. Therefore, these alternatives should be more explored, to build consistent counter stainings to be used from early to later stages.

4.2.2. Mounting Position Comparison

Position is an important variant to take in account while mounting embryos and larvae with the objective of acquiring images through the whole brain to generate comprehensive atlases. Position should be comparable during development but also should allow to image the whole brain. At early stages, such 1 and 2 dpf, embryos show a curvature between the forebrain and midbrain. This curvature (that is not present in later stages) constitutes a challenge to image the whole brain. Furthermore, deep tissue imaging is still challenging, since there is more light scattering deeper in the Z axis. By positioning the

embryos differently from the conventional way of mounting and using different microscopes it is possible to achieve the whole brain imaging.

The conventional way of mounting – Horizontal Position (*Figure 2.1.A*) – is reached by making embryos and larvae to stand with their dorsal side directly in contact with the coverslip. This way of mounting is used since it's closer to the way of mounting in later stages, making it more comparable. Even though this is true, horizontal positioning makes the forebrain less visible and the hindbrain more visible, which in some cases might create a challenge to annotation. The alternative way of mounting in the confocal microscope – Vertical Position (*Figure 2.1.B)* – is done by putting the rostral part of the head in contact with the coverslip. This makes the forebrain more available to image. This suggests that both ways of mounting complement each other, making the annotation process easier, since they both combine a good imaging of the forebrain and hindbrain, overlapping the midbrain.

On one hand, the confocal microscope used has the better resolution, but on the other hand, the same sample cannot be mounted easily twice, not being possible yet to have the same embryo in horizontal and vertical positions. In this way, we explored light sheet microscopy that allows to reposition the same embryo countless times until reaching the "right" position (*Figure 2.2*). With this equipment, it is possible to image the same brain regions as with the confocal microscope but also image more rostral or dorsal regions. This is a great advantage to annotate regions that are not commonly accessible through confocal. The main disadvantage with the light sheet imaging is that there is less resolution than in the confocal imaging, making it a challenge for registration. This suggests that the light sheet image can be a complement for confocal imaging, since moving the same sample while imaging facilitates annotation of "difficult to distinguish" brain regions.

5. Conclusion and Future Perspectives

In conclusion, the use of transgenic lines to express GFP in certain neuronal populations through the GFF:UAS system is accurate and useful for characterizing anatomically these lines. The anatomical characterization during the early days of development contributes to the enrichment of existing anatomical atlases with advanced stages of development. Consequently, this will have an impact on how future experiences will be generated and how neural networks will be understood.

Here we consider that our project – Anatomical Characterization of the Pitx2c:GFF and the sst1:GFF Transgenic Lines (Orger lab) Through Development – was successfully achieved. Although there are some limitations to our method, we performed the anatomical characterization with some level of confidence. In addition, we believe that the second part of the project – Establishing a Reference Marker and Sample Orientation for the Generation of a Brain Atlas for the 2dpf Zebrafish Embryo – was successful. Exploring alternatives, in counter staining or mounting methods and imaging equipment, is needed in this area to improve and facilitate the characterization of these early stages, which have not thoroughly been explored.

In the future, it would be worthwhile to perform the image registration process in order to input the data obtained in the pre-existing atlases (at least for 6dpf). It would also be good to further test the counter staining alternatives (trying to find that one suitable for early stages) and apply the new mounting methods (to understand if they can be useful in the registration process).

Thus, we believe that our contribution can be beneficial for designing future studies, either with the lines that we have characterized, or the new alternative methods we have presented here.

6. Bibliography

1. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013;496(7446):498–503.

2. Orger MB. The Cellular Organization of Zebrafish Visuomotor Circuits. Curr Biol 2016;26(9):R377–85.

3. Mueller DT, Wullimann M. Atlas of Early Zebrafish Brain Development: A Tool for Molecular Neurogenetics [Internet]. 2015. Available from: https://books.google.de/books?id=5dScBAAAQBAJ

4. Alunni A, Coolen M, Foucher I, Bally-Cuif L. Chapter 32 - Neurogenesis in Zebrafish [Internet]. In: Rubenstein JLR, Rakic PBT-P and CTS in the DCNS and PNS, editors. . Oxford: Academic Press; 2013. page 645–77.Available from: http://www.sciencedirect.com/science/article/pii/B9780123972651000691

5. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn an Off public 1995;203(3):253–310.

6. Streit A, Berliner AJ, Papanayotou C, Sirulnik A, Stern CD. Initiation of neural induction by FGF signalling before gastrulation. Nature [Internet] 2000;406:74. Available from: http://dx.doi.org/10.1038/35017617

7. Wilson S, Rydström A, Trimborn T, Willert K, Nusse R, Jessell TM, et al. The status of Wnt signalling regulates neural and epidermal fates in the chick embryo. Nature [Internet] 2001;411:325. Available from: http://dx.doi.org/10.1038/35077115

8. Okuda Y, Ogura E, Kondoh H, Kamachi Y. B1 SOX Coordinate Cell Specification with Patterning and Morphogenesis in the Early Zebrafish Embryo. PLOS Genet [Internet] 2010;6(5):e1000936. Available from: https://doi.org/10.1371/journal.pgen.1000936

9. Schmidt R, Strähle U, Scholpp S. Neurogenesis in zebrafish - from embryo to adult. Neural Dev 2013;8(1):1–13.

10. Schmidt R, Strähle U, Scholpp S. Neurogenesis in zebrafish – from embryo to adult. Neural Dev [Internet] 2013;8(1):3. Available from: https://doi.org/10.1186/1749-8104-8-3

11. Appel B, Chitnis A. Neurogenesis and Specification of Neuronal Identity BT - Pattern Formation in Zebrafish [Internet]. In: Solnica-Krezel L, editor. . Berlin, Heidelberg: Springer Berlin Heidelberg; 2002. page 237–51.Available from: https://doi.org/10.1007/978-3-540-46041-1_12

12. Korzh V, Strähle U. Proneural, prosensory, antiglial: the many faces of neurogenins. Trends Neurosci [Internet] 2002;25(12):603–5. Available from: http://dx.doi.org/10.1016/S0166-2236(02)02275-0

13. Baye LM, Link BA. Nuclear Migration During Retinal Development. Brain Res [Internet] 2008;1192:29–36. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2674389/

14. Haddon C, Smithers L, Schneider-Maunoury S, Coche T, Henrique D, Lewis J. Multiple delta genes and lateral inhibition in zebrafish primary neurogenesis. Development [Internet] 1998;125(3):359 LP – 370. Available from: http://dev.biologists.org/content/125/3/359.abstract

15. Dong Z, Yang N, Yeo S-Y, Chitnis A, Guo S. Intralineage Directional Notch Signaling

Regulates Self-Renewal and Differentiation of Asymmetrically Dividing Radial Glia. Neuron [Internet] 2012;74(1):65–78. Available from: http://dx.doi.org/10.1016/j.neuron.2012.01.031

16. Borodinsky LN, Belgacem YH, Swapna I, Visina O, Balashova OA, Sequerra EB, et al. Spatiotemporal integration of developmental cues in neural development. Dev Neurobiol [Internet] 2015;75(4):349–59. Available from: https://www.ncbi.nlm.nih.gov/pubmed/25484201

17. Blader P, Strähle U. Zebrafish developmental genetics and central nervous system development. 2000.

18. Kalueff A V, Gebhardt M, Stewart AM, Cachat JM, Brimmer M, Chawla JS, et al. Towards a Comprehensive Catalog of Zebrafish Behavior 1.0 and Beyond. Zebrafish [Internet] 2013;10(1):70–86. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3629777/

19. Mitsuhiro H, Masahiko H. Development and evolution of cerebellar neural circuits. Dev Growth Differ [Internet] 2012;54(3):373–89. Available from: https://doi.org/10.1111/j.1440-169X.2012.01348.x

20. Easter Jr SS, Nicola GN. The Development of Vision in the Zebrafish. Dev Biol [Internet] 1996;180(2):646–663. Available from: https://ac.els-cdn.com/S0012160696903358/1-s2.0-S0012160696903358-main.pdf?_tid=5127746c-bbc2-4d6b-99ba-76814082f1d5&acdnat=1549017063_e3b9adb656fb244c4fe9fc5d7a0f7c72%0Ahttp://www.sciencedirect.com/science/article/pii/S0012160696903358

21. Huang YY, Neuhauss SCF. The optokinetic response in zebrafish and its applications. Front Biosci 2008;13(5):1899–916.

22. Portugues R, Feierstein CEE, Engert F, Orger MBB. Whole-Brain Activity Maps Reveal Stereotyped, Distributed Networks for Visuomotor Behavior. Neuron [Internet] 2014;81(6):1328–43. Available from: http://www.sciencedirect.com/science/article/pii/S0896627314000506

23. Severi KE, Portugues R, Marques JC, O'Malley DM, Orger MB, Engert F. Neural Control and Modulation of Swimming Speed in the Larval Zebrafish. Neuron [Internet] 2014;83(3):692–707. Available from: http://www.sciencedirect.com/science/article/pii/S0896627314005789

24. Asakawa K, Kawakami K. Targeted gene expression by the Gal4-UAS system in zebrafish. Dev Growth Differ 2008;50(6):391–9.

25. Asakawa K, Suster ML, Mizusawa K, Nagayoshi S, Kotani T, Urasaki A, et al. Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. Proc Natl Acad Sci U S A 2008;105(4):1255–60.

26. Garric L, Ronsin B, Roussigné M, Booton S, Gamse JT, Dufourcq P, et al. Pitx2c ensures habenular asymmetry by restricting parapineal cell number. Development [Internet] 2014;141(7):1572–9. Available from: https://www.ncbi.nlm.nih.gov/pubmed/24598158

27. Essner JJ, Branford WW, Zhang J, Yost HJ. Mesendoderm and left-right brain, heart and gut development are differentially regulated by pitx2 isoforms. Development [Internet] 2000;127(5):1081 LP – 1093. Available from: http://dev.biologists.org/content/127/5/1081.abstract

28. Wolman MA, Sittaramane VK, Essner JJ, Yost HJ, Chandrasekhar A, Halloran MC. Transient axonal glycoprotein-1 (TAG-1) and laminin-alpha1 regulate dynamic growth cone behaviors and initial axon direction in vivo. Neural Dev [Internet] 2008;3:6. Available from: https://www.ncbi.nlm.nih.gov/pubmed/18289389

29. Devos N, Deflorian G, Biemar F, Bortolussi M, Martial JA, Peers B, et al. Differential expression of two somatostatin genes during zebrafish embryonic development. Mech Dev [Internet] 2002;115(1):133–7. Available from: http://www.sciencedirect.com/science/article/pii/S0925477302000825

30. Miguel B, Madeira N De. Developmental characterization of neuronal subpopulations involved in zebrafish visual processing. 2017;

31. Randlett O, Wee CL, Naumann EA, Nnaemeka O, Schoppik D, Fitzgerald JE, et al. Whole-brain activity mapping onto a zebrafish brain atlas. Nat Methods [Internet] 2015;12(11):1039–46. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4710481/

32. Marquart GD, Tabor KM, Brown M, Strykowski JL, Varshney GK, LaFave MC, et al. A 3D Searchable Database of Transgenic Zebrafish Gal4 and Cre Lines for Functional Neuroanatomy Studies. Front Neural Circuits [Internet] 2015;9(November):1–17. Available from: http://journal.frontiersin.org/Article/10.3389/fncir.2015.00078/abstract

33. Kunst M, Laurell E, Mokayes N, Kramer A, Kubo F, Fernandes AM, et al. A Cellular-Resolution Atlas of the Larval Zebrafish Brain. Neuron [Internet] 2019;103(1):21-38.e5. Available from: http://www.sciencedirect.com/science/article/pii/S0896627319303915

34. Löhr H, Ryu S, Driever W. Zebrafish diencephalic A11-related dopaminergic neurons share a conserved transcriptional network with neuroendocrine cell lineages. Development [Internet] 2009;136(6):1007 LP – 1017. Available from: http://dev.biologists.org/content/136/6/1007.abstract

35. Löhr H, Hess S, Pereira MMA, Reinoß P, Leibold S, Schenkel C, et al. Diet-Induced Growth Is Regulated via Acquired Leptin Resistance and Engages a Pomc-Somatostatin-Growth Hormone Circuit. Cell Rep [Internet] 2018;23(6):1728–41. Available from: http://www.sciencedirect.com/science/article/pii/S2211124718305473

36. Ronneberger O, Liu K, Rath M, Rue D, Mueller T, Skibbe H, et al. ViBE-Z: A framework for 3D virtual colocalization analysis in zebrafish larval brains. Nat Methods 2012;9(7):735–42.

37. Marquart GD, Tabor KM, Horstick EJ, Brown M, Geoca AK, Polys NF, et al. High-precision registration between zebrafish brain atlases using symmetric diffeomorphic normalization. Gigascience [Internet] 2017;6(8):1–15. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5597853/

38. Velez MR. Generation of new transgenic zebrafish lines for studying neuronal circuits underlying behavior in zebrafish. 2018;

39. Affaticati P, Le Mével S, Jenett A, Rivière L, Machado E, Mughal BB, et al. X-FaCT: Xenopus-Fast Clearing Technique BT - Xenopus: Methods and Protocols [Internet]. In: Vleminckx K, editor. . New York, NY: Springer New York; 2018. page 233–41.Available from: https://doi.org/10.1007/978-1-4939-8784-9_16

40. Cooper M, Szeto D, Sommers-Herivel G, Topczewski J, Solnica-Krezel L, Kang H-C, et al. Visualizing morphogenesis in transgenic zebrafish embryos using BODIPY TR methyl ester dye as a vital counterstain for GFP. Dev Dyn 2005;232:359–68.

41. Martins S, Monteiro JF, Vito M, Weintraub D, Almeida J, Certal AC. Toward an Integrated Zebrafish Health Management Program Supporting Cancer and Neuroscience Research. Zebrafish [Internet] 2016;13(S1):S-47-S-55. Available from: http://online.liebertpub.com/doi/10.1089/zeb.2015.1198

42. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012;9(7):676.

43. Bianco IH, Wilson SW. The habenular nuclei: a conserved asymmetric relay station in the vertebrate brain. Philos Trans R Soc Lond B Biol Sci [Internet] 2009;364(1519):1005–20. Available from: https://www.ncbi.nlm.nih.gov/pubmed/19064356

44. Bindels DS, Haarbosch L, van Weeren L, Postma M, Wiese KE, Mastop M, et al. mScarlet: a bright monomeric red fluorescent protein for cellular imaging. Nat Methods [Internet] 2017;14(1):53–6. Available from: https://doi.org/10.1038/nmeth.4074