ZEBRAFISH GLIOMA XENOGRAFT MODELS: *IN VIVO* INVESTIGATION OF INJECTION METHODS AND DEVELOPMENT OF A STREAMLIT APPLICATION

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September 2023

We certify that we have read this thesis and that, in our opinion, it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

ZEBRAFISH GLIOMA XENOGRAFT MODELS: *IN VIVO* INVESTIGATION OF INJECTION METHODS AND DEVELOPMENT OF A STREAMLIT APPLICATION

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M.Sc. in Neuroscience

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September 2023

Glioblastoma (GBM) is one of the most aggressive and lethal forms of primary brain cancer, posing significant challenges to effective treatment and patient outcomes. Despite extensive research efforts, our understanding of GBM biology and the development of novel therapeutic strategies remains limited. Zebrafish xenograft models have emerged as a promising tool in cancer research, offering unique advantages in studying GBM. This thesis explores the utility of zebrafish xenograft models in advancing our understanding of GBM. Due to their genetic and physiological similarities to humans, zebrafish provide an excellent platform for studying GBM pathogenesis, tumor progression, and drug screening. Their transparency during early development allows for real-time visualization of tumor growth, invasion, and response to treatments. Moreover, zebrafish models enable rapid and cost-effective high-throughput drug screening, accelerating the identification of potential GBM therapeutics. In this thesis, I focused on creating an application named ZenofishDb Glioma, which is a more evolved and focused

version of our previous database called ZenofishDb. ZenofishDb Glioma has been created using Python Streamlit, and comprises only the glioma studies and uses Natural Language Processing (NLP) to classify better, and effectively find and summarize information about zebrafish glioma xenograft models. In addition, after searching ZenofishDb Glioma, I decided to investigate an experimental protocol for injection of glioblastoma cells in the zebrafish model to test effects of injected cell numbers. Using MGG-119-GFP cells and Casper zebrafish, I injected different numbers of cells at different locations and stages, i.e., blastula and 2 days post fertilization, and observed there were significant differences between groups at 5 dpf using multiple quantification strategies. In conclusion, ZenofishDb Glioma can help design effective xenotransplantation strategies and make comparisons to understand how different experimental parameters affect the outcome of zebrafish glioma xenograft models.

Keywords: Brain cancer, glioblastoma, glioma, zebrafish, xenograft, Streamlit, Phyton, application.

ÖZET

ZEBRA BALIĞI GLIOMA KSENOGRAFT MODELLERİ: ENJEKSİYON YÖNTEMLERİNİN *IN VIVO* ARAŞTIRILMASI VE BİR STREAMLIT APLİKASYONUNUN GELİŞTİRİLMESİ

Rüya Tombuloğlu

Nörobilim, Yüksek Lisans

Tez Danışmanı: Özlen Konu Karakayalı

Eylül 2023

Glioblastoma (GBM), primer beyin kanserinin en agresif ve ölümcül formlarından biridir ve etkili tedavi bulunması ve hasta sağlığının takibi açısından önemli zorluklar teşkil etmektedir. Kapsamlı araştırma çabalarına rağmen, GBM biyolojisi ve yeni terapötik stratejilerin geliştirilmesi konusundaki anlayışımız sınırlı kalmaktadır. Zebra balığı ksenograft modelleri, kanser araştırmalarında umut verici bir araç olarak ortaya çıkmıştır ve GBM'nin incelenmesinde benzersiz avantajlar sunmaktadır. Bu tez, GBM anlayışımızı ilerletmede zebra balığı ksenograft modellerinin faydasını araştırmaktadır. İnsanlarla olan genetik ve fizyolojik benzerlikleri nedeniyle zebra balığı, GBM patogenezi, tümör ilerlemesi ve ilaç taramasını incelemek için mükemmel bir platform sağlar. Erken gelişim sırasındaki şeffaflıkları, tümör büyümesinin, istilasının ve tedavilere yanıtın gerçek zamanlı olarak görselleştirilmesine olanak tanır. Dahası, zebra balığı modelleri hızlı ve uygun maliyetli yüksek verimli ilaç taramasına olanak tanıyarak potansiyel GBM terapötiklerinin belirlenmesini hızlandırır. Bu tezde, ZenofishDb adlı önceki

veritabanımızın daha gelişmiş ve odaklanmış bir versiyonu olan ZenofishDb Glioma adlı bir uygulama oluşturmaya odaklandım. ZenofishDb Glioma, Python Streamlit kullanılarak oluşturuldu ve yalnızca glioma çalışmalarını içeriyor ve daha iyi sınıflandırmak ve zebra balığı glioma xenograft modelleri hakkındaki bilgileri etkili bir şekilde bulmak ve özetlemek için Doğal Dil İşleme (DDİ) kullanıyor. Ek olarak, ZenofishDb Glioma'yı araştırdıktan sonra, enjekte edilen hücre sayılarının etkilerini test etmek için zebra balığı modelinde glioblastoma hücrelerinin enjeksiyonu için deneysel bir protokol araştırmaya karar verdim. MGG-119-GFP hücreleri ve Casper zebra balığı kullanarak, farklı konum ve aşamalarda, yani blastula ve döllenmeden 2 gün sonra, farklı sayıda hücre enjekte ettim ve çoklu kantifikasyon stratejileri kullanarak 5 dpf'de gruplar arasında önemli farklılıklar olduğunu gözlemledim. Sonuç olarak, ZenofishDb Glioma etkili ksenotransplantasyon stratejileri tasarlamaya ve farklı deneysel parametrelerin zebra balığı glioma ksenograft modellerinin sonucunu nasıl etkilediğini anlamak için karşılaştırmalar yapmaya yardımcı olabilir.

Keywords: Beyin kanseri, glioblastoma, glioma, zebra balığı, zenograft, Streamlit, Phyton, aplikasyon.

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Chapter 1

Introduction

1.1 Brain Cancer

Brain tumors occur due to the uncontrollable division of the cells of the central nervous system. Depending on the division speed and metastatic properties, they can be grouped into benign or malignant tumors. Although benign tumors are non-cancerous and divide slowly, they may pose a serious problem and require medical treatment. On the other hand, malignant tumors are cancerous, metastatic, and aggressive, causing death worldwide. According to GLOBOCAN 2020, there were 19.3 million emerging incidents causing 10 million deaths due to various cancers, and malignant brain tumors hold the 13th position as the leading cause of mortality among all cancers in 2020 [1].

Brain tumors can be classified as either primary or secondary, depending on their origin. Primary brain tumors stem from the central nervous system - brain and spinal cord - and occur less frequently than secondary tumors [2]. Although there are more than 120 types of primary CNS tumors, according to the type of tissue they originate, they can be categorized into gliomas - tumors that originate in glial cells; meningiomas - tumors that originate in the meninges; and pituitary tumors - tumors that originate in the pituitary gland [3]. Secondary brain tumors develop outside of the central nervous system and metastasize to the brain and are the most

common tumor type. Predominantly, the primary source of secondary brain tumors is the liver, breast, skin, and gastrointestinal tract [4].

Brain tumors can cause a wide range of symptoms, from headaches and seizures to loss of control of the limbs and understanding a language, depending on the tumor size, location, whether it is benign or malignant, as well as biomarkers and mutations it carries. The current diagnosis of brain tumors comprises a combination of physical examination, biopsy, and, most commonly, CT and MRI scans [5]. Although most benign tumors don't pose a significant threat to health with regular check-ups, their development should be followed. Unfortunately, there isn't any permanent cure for the disease; alternative treatments to surgery, radiation, chemotherapy, and medication therapy are being searched.

1.1.1 Glioma and Glioblastoma

Gliomas are one of the most common primary brain tumors that occur in the glial cells of the brain and the spinal cord. Depending on the type of glial cell they stem from, they can be called astrocytoma, ependymoma, or oligodendroglioma. Astrocytomas originate in astrocytes, which keep homeostasis balanced by regulating synaptic transmission [6]. As a healthy glial cell, especially in astrocytoma, turns into a cancerous stage, it tends to extend its projections to infiltrate other brain regions [7]. This spreading behavior is said to be one of the defense mechanisms of astrocytes against chemo and radiation therapy [8]. Menard et al. hypothesize that glutamate dysregulation within a neuron causing an aberrant release of the neurotransmitter can cause a neurotoxic response resulting in the retraction of neighboring neurons, which allows astrocytic projections to migrate [8]. Also, glutamate-induced Ca^{2+} rise in astrocytomas may

increase filopodiagenesis in grade 2 astrocytomas [8], [9]. Grading of astrocytomas ranges from 1-4, while grade 1 tumors, pilocytic astrocytoma, are benign and can be removed by resection as the tumor grade increases; its lethality also does. Grade 2 tumors are called diffuse astrocytomas and are considered low-grade infiltrative tumors, while grade 3 anaplastic astrocytomas show a much more aggressive phenotype. Finally, grade 4 glioblastomas are considered the deadliest, with the worst prognosis around one year after diagnosis [10].

Oligodendrogliomas originate in the oligodendrocytes, showing a trademark of co-deletion of 1p and 19q chromosomal arms and IDH 1 or IDH 2 mutation [11]. Although they are not as deadly as glioblastomas, which comprise around 60% of all infiltrative gliomas, they can occur together with glioblastomas (GBM-O) [12]. The World Health Organization (WHO) introduced this new sub-type, which shows oligodendroglial and astrocytic differentiation to better classify glioblastomas [10]. Glioblastomas can be divided into three main classes as: primary, secondary, and de novo. While primary glioblastomas are more aggressive and have higher occurrence than secondary glioblastomas, they also show different genetic characteristics, such as EGFR amplification and PTEN deletion [13], [14]. Whereas secondary glioblastomas have a better prognosis and generally show markers like TP53 and IDH1 mutations [15]–[18].

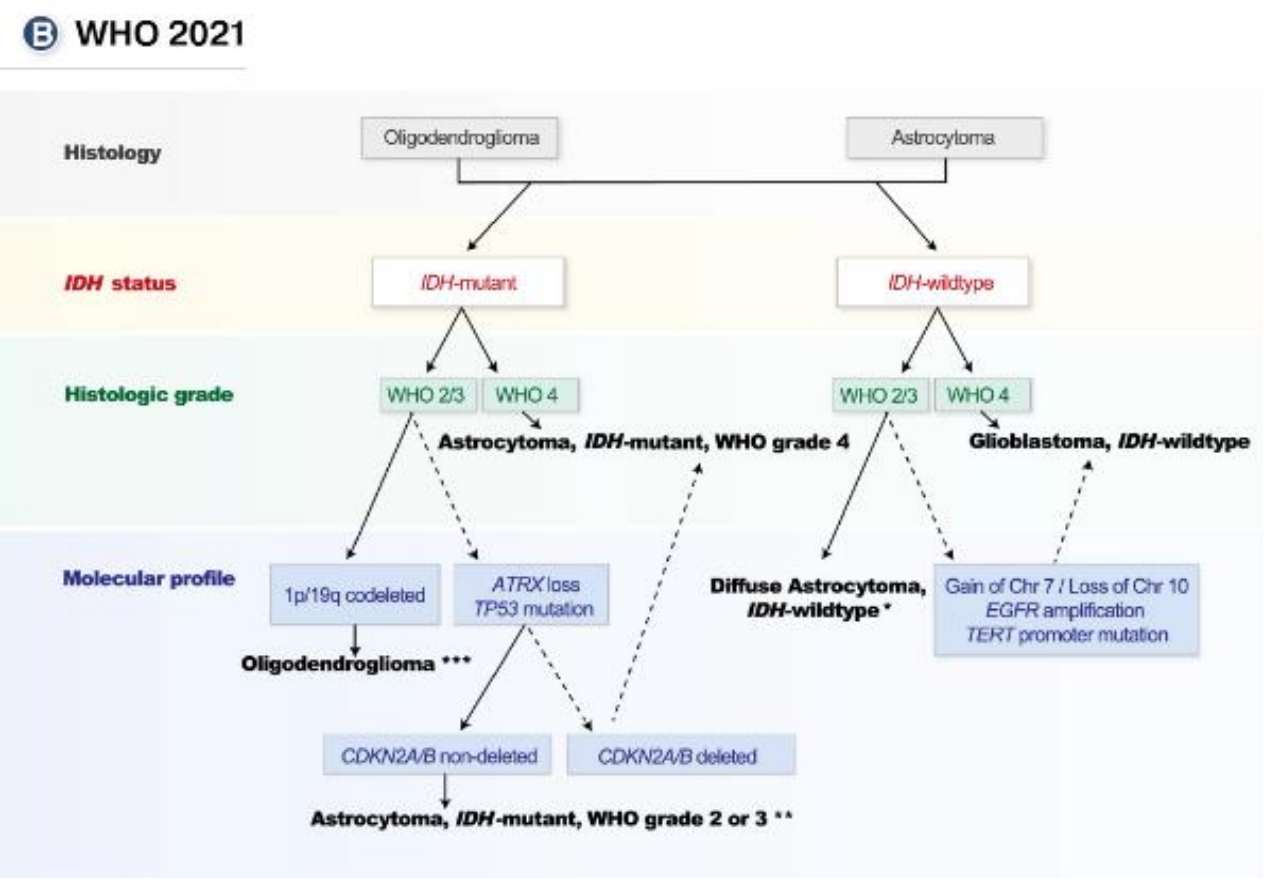


Figure 1.1 1 WHO 2021 classification of brain tumors (From [19], Licence Number 5652670367920).

1.1.2 Current Treatment of Glioblastoma

To this day, glioblastomas remain one of the deadliest cancer types with no cure. Current glioma and glioblastoma treatment offers a mixture of radiotherapy and chemotherapy and an oral drug called Temozolomide (TMZ) if surgical removal of the tumor is not adequate [20]. Unfortunately, these methods only offer preventive solutions and not a final ending for the disease due to its heterogeneous and drug-resistant environment. Moreover, because of how glioblastoma

infiltrates within the brain, surgical removal of the tumorous tissue is not always an option [21]. Moreover, even with prolonged radiation and chemotherapies, cancer recurs almost always due to its aggressiveness and drug-resistant nature [22], [23]. Knowing that glioblastoma is the most common primary brain cancer, constituting 80% of all primary brain cancers with a median survival of 12-18 months after diagnosis, treating glioblastomas requires an urgent call on therapeutic research of the disease [24]–[26].

1.1.3 Alternative Treatment for Glioblastoma

Considering current glioblastoma treatments' inadequate and ineffective approaches, it is essential to develop multidisciplinary approaches incorporating translational and personalized medicine and precision care for each patient. One of the promising alternatives in brain cancer treatment is the use of vaccines. Since tumor cells suppress the immune system, vaccine strategies such as tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs) can offer re-sensitization of the immune system against the specific target [27]. Research by Phuphanich et al. shows that a cocktail of TAAs such as HER2, MAGE-1, and IL13Rα2 increases the overall median survival in newly diagnosed GBM patients [28]. Another personalized alternative for the treatment of glioblastomas utilizes the help of retroviral replicating vectors (RRVs). RRVs offer a long-term solution as they permanently incorporate into the tumor tissue without being eliminated from the microenvironment [29]**.** Multiple encouraging findings are using Toca 511, a gamma retroviral replicating vector encoding cytosine deaminase, to transduce tumor cells with the gene cytosine deaminase for targeted drug administration [30], [31]. Once flucytosine is administered systematically, only the transduced cells can metabolize this prodrug into its active form, 5-fluorouracil – a cytotoxic chemotherapeutic used in different cancers [30]–[32]. As every

patient has their own contributing factor to how their tumor grows, utilizing personalized medicine is vital in figuring out a cure for cancer. Considering the genetic diversity causing different brain cancers, a high-throughput utilization of personalized medicine is needed. In addition to the above-mentioned alternatives, in vivo models such as mice and zebrafish serve as great options, especially in xenograft studies and drug discovery research [33], [34].

1.2 Zebrafish as a Model for Brain Cancer Research

As the need for high-throughput research in cancer studies increases daily, zebrafish has emerged as a low-cost and efficient organism model to study heterogeneous and fast-evolving microenvironments of different cancers. Considering 82% of the zebrafish orthologs are related to human diseases offers significant genetic conservation between these two vertebrate species, allowing a parallel/comparative research environment [35], [36]. Aside from their fast-speed developmental period and high fecundity, zebrafish have become especially useful in imaging studies due to their transparent and translucent features [37]. Vasculature imaging is crucial in brain cancer since most CNS malignancies show invasive properties. Real-time imaging of the vasculature is critical for understanding the metastasis niche and contributing factors to metastasis. For instance, transgenic zebrafish lines expressing a fluorescent protein in their blood vessels, such as Tg(fli1a: GFP) zebrafish, can be utilized to observe metastatic and invasive properties of the tumor over time [38]–[40]. Not only has zebrafish been a good model for its openness for genetic manipulation and its transgenic properties, but it is also a popular go-to in vivo organism for high-throughput drug research. Ridges et al., for example, used zebrafish to screen 26400 small molecules against leukemia and found a promising lead – Lenaldekar, which

can destroy immature T-cells without disrupting the cell cycle of the zebrafish [41]. When combined with xenotransplantation, zebrafish can offer a relatively low-cost and fast drug screening environment compared to murine models.

1.2.1 Zebrafish Xenograft Models

Xenograft or xenotransplantation transfers cells, tissues, or organs from one species to another. So far, different malignant cells have been transplanted into zebrafish to study different cancers such as leukemia, gastric carcinoma, and neuroendocrine-system-originated tumors [42]–[44]. Whether the transplant is a cell line or patient-derived xenograft (PDX), mouse xenografts were the primary model to study human cancers for an extended period. However, considering the amount of primary tissue that needs to be collected for large-scale, replicable xenograft research and the time required for mice to be used in the transplantation study limit the speed of the procedure [45]. On the other hand, zebrafish offers a low-cost, high-throughput, and fast xenotransplantation option as its developmental period allows tumors to be injected and observed in a few days [46]. Moreover, since the tumor can also be injected into embryonic or larval zebrafish, the transparent and translucent properties of the zebrafish provide easy handling and imaging conditions during and after the injection.

Moreover, PDXs are excellent models as they mimic a patient's tumor microenvironment better than cell lines since they are not as differentiated as cell lines. One recent study by Almstedt et al. shows the use of GFP-tagged patient-derived glioblastoma xenograft in real-time tumor progression analysis. They show that all injection models had different survival, growth and invasion patterns and showed parallelism to their mouse xenograft counterparts [47]. These

findings not only indicate that zebrafish can be an alternative in vivo organism to mouse, but also emphasizes it can be the means to test personalized medicine in the treatment of glioblastomas. Another group of scientists has utilized zebrafish transplantation to create an orthotopic model of human pediatric high-grade glioma to screen for drugs. Accordingly, after 2 days post fertilization (dpf) and the injection into the forebrain, they started the TMZ, VPA, and decitabine treatments alone and in combination and showed that in all cases, the cell number decreased [48].

1.2.1.1 Blastula vs. yolk xenograft models

The Blastula stage of zebrafish refers to the period between 2.25 – 5.25 hpf. In this period, cells divide mitotically, and, starting from the 128-cell stage, they increase in size fast. During the blastula stage, a critical period called mid-blastula transition (MBT) occurs. This marks the transition period of zebrafish from blastula to gastrula. Before the MBT, cells of the embryo divide fast and synchronously.

Moreover, embryos only use maternal mRNA for their growth since zygotic transcription is repressed by mechanisms such as methylation [49]. So, the cell cycle is quite short - without G1 and G2 stages, and zygotic DNA is not used [50], [51]. With the initiation of mid-blastula transition, which happens in the 10^{th} cycle of the embryonic divisions, zygotic transcription gets activated [52]. Now, the zygote starts to produce its own mRNA, and maternal transcripts are no longer in use [53]. This change adds G1 and G2 stages and the cell cycle lengthens. Cells also start to divide less meta-synchronously.

Experiments with blastula and yolk injections can provide insights into different developmental processes and tumor models. While blastula xenografts are often used to investigate early

developmental processes such as different tissue type formation, yolk xenografts allow investigation of later stages of the zebrafish, such as organogenesis and tissue differentiation. Zebrafish yolk acts as a food source during early development and completely gets depleted at the end of 5 dpf, leaving the larva to be dependent on external feeding [54]. Zebrafish larva is considered to be fully developed at the end of 3 dpf [55]. Considering these critical time points, migration and proliferation of the tumor can be affected based on its injection time and site.

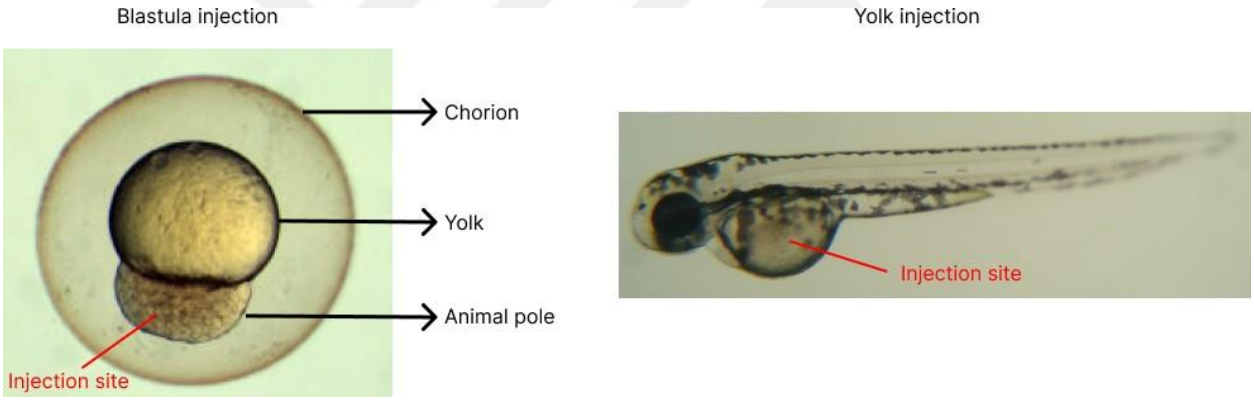


Figure 1.2 1 Injection models for blastula and 2 dpf stage zebrafish. Approximately 4 hpf blastula stage embryo (on the left). 2 dpf stage larva (on the right).

1.2.1 High-throughput Drug Screening in Zebrafish

High-throughput drug screening allows the discovery and optimization of relevant compounds out of large pools in an automated way. When combined with zebrafish's fast development and high fecundity rate, testing different drugs can be achieved in a cost-efficient way. Finding a novel therapeutic from scratch generally takes about 15 years on average, considering all the

steps from research and development to drug approval for marketing [56]. That's why the current research movement in drug discovery flows in a direction where drug repurposing and combinatorial use of multiple drugs are predominant. For such a movement to further develop, lots of drug panels consisting of already-approved and still-in-trial compounds need to be screened in a high-throughput manner. Both in vitro and in vivo screens can yield the best results for coming up with an alternative treatment or even a cure for a rare disease (Table 1.2).

Moreover, the application of drug screening after a xenograft model can offer preliminary results about drugs' mechanisms. For instance, we can see in Cheng et al.'s research that after they screened 79 drugs in glioblastoma cell lines, they repurposed an antipsychotic drug – thioridazine as a promising anti-glioblastoma agent as it killed glioblastoma stem cells (GSCs) [57]. TMZ is one of the most tested drugs in brain cancer research as it is already FDA-approved and shows cancer-slowing effects. So, most scientists are trying to develop combinatorial therapies to find an alternative to glioblastoma using TMZ. One study shows that when given with Onalespib, TMZ's effect on tumor burden can be seen even more drastically. The authors used the U251 glioblastoma line to intracranially inject into the larval midbrain to observe a synergistic effect of the drugs and found that tumor size shrank even more when combinatorially given as compared to both drugs were given separately [58].

Table 1.1 1 Selected publications testing high-throughput drug screening

Panel name	**Human Cell Lines**	**In vivo model**	**Reference**
NIH clinical collection (NCC) that includes 446 FDA-approved drugs	U87, LN443, A172, U118, SK72, SK262, SK429 and SK660		[59]
Custom-made	GBM8401 cells and U87MG cells		[57]
(NINDS) custom collection II library	U87 human glioma cells and HEK 293T human fibroblast cells		[60]
BioMol (Enzo Life Sciences), National Institutes of Health (NIH) Clinical Collection, National Cancer Institute/NIH Developmental Therapeutics Program Approved Oncology Drugs Set, Sigma Aldrich, and Tocris Biosciences	GBM cell lines U87MG, U343MG, U373MG, A172, and T98G.		[61]
MicroSource Spectrum collection	Tumor cells from patients	mice xenograft	[62]
Custom-made	U87, U251, and 293T cells	Zebrafish xenograft	[63]

provided by NCI's Developmental Therapeutics Program and consists of 147 FDA-approved cancer therapy agents (AOD IX)	6 IDHmut glioma cell lines		[64]
The oncology drug collection consisted of 461 compounds	12 patient-derived primary GBMs cultured as tumorspheres		[65]
The Approved Oncology Drugs Set VI (National Cancer Institute), comprising 129 drugs	RIG cell lines MAF-145 and MAF-496		[66]

1.3 Python/Streamlit

1.3.1 Structure of Python/Streamlit Applications

Streamlit is an open-source and free Python framework that helps people create web applications (https://streamlit.io/). It is a Python-based library that is compatible with other major Python libraries such as scikit, NumPy, pandas, and Matplotlib, allowing especially machine learning- and data science-based web apps to be developed. Compared to other frameworks such as Django or Flask, Streamlit provides an easy-to-use module and doesn't require knowledge of CSS, HTML, or Javascript [67]. Streamlit applications run from top-to-bottom, and every time a

script is executed, meaning a tab pointing to the app is opened, it reruns in the same manner. Since it allows user interaction, whenever a user makes a change in the widgets, the script is rerun, and the output of the new value is projected during the run [68], [69]. There are lots of web apps harboring the power of Streamlit in different areas, such as computer vision, education, finance, business, NLP, and language. It has become a popular choice of framework as it allows fast and direct deployment of the app without writing backend codes or handling HTTP requests [70]. While there are multiple platforms to create web applications, especially when compared to Shiny, due to Python's general-purpose nature compared to R's exclusive focus on data analytics, web applications created using Streamlit possess greater potency and offer simplified scalability for production environments when contrasted with applications developed using Shiny [71], [72].

1.3.2 Natural Language Processing (NLP)

NLP is the intersection set of artificial intelligence, computer science, and human linguistics, concerned with machines learning and processing human language as well as humans can [73]. The goal of NLP mostly involves analyzing spoken or written text, such as extracting information from a text grouping, classifying, or categorizing texts or a certain part of a text [74]. This technology allows machines to do tasks that humans fall short of or are incapable of. The evolution of NLP can be divided into three main categories: symbolic NLP, statistical NLP, and neural NLP. In the early years of natural language processing, symbolic NLP was mostly about automatic translation and chatterbots such as PARRY, Jabberwacky, and Racter [75], [76]. But, with the need for a more quantitative evaluation of human language, its progress shifted to a more statistical era. With statistical NLP, machine learning algorithms specifically for language processing were created, such as supervised, semi-supervised, or unsupervised models [77]. This

computational power provided the roots for neural NLP to emerge as representation learning and deep neural networks have started to be used in medicine and healthcare sectors [78].

Table 1.2 1 List of Phyton packages used in the creation of the Streamlit app

Name	**Reference**
streamlit	1.14.1
streamlit_option_menu	0.3.2
st_aggrid	0.3.3
pandas	1.5.1
NumPy	1.23.4
matplotlib.pyplot	3.6.2
altair	4.2.0
wordcloud	1.8.2.2
pdfminer.high_level	V20191125
gensim.summarization	3.8.2
urllib.request	1.26.12
metapub	0.5.5
fitz	No package description

1.4 Aims and Rationale

Previously, our lab has published ZenofishDb v1.1, a general database that houses xenograft-related studies in zebrafish. However, this version of Zenofish does not have natural language processing, limiting its use to gather data from the results of a publication or a related set of publications. In this thesis, my first aim was to develop a new version of ZenofishDb focused on Glioma/Glioblastoma to incorporate novel features and provide a prototype for tissue-specific xenograft databases in zebrafish. My second aim was to compare the differences between injection methodology based on injection site and injected cell volume using glioma cells that are xenografted zebrafish using blastula injections and test if blastomere injections were different than the yolk injections.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Laboratory Reagents and Kits

General laboratory reagents and kits used in the lab are listed in Table 2.1 below with their respective catalog number and company name.

Table 2.1 1 General laboratory products and kits used for the experiments.

Product Name	Catalog Number	Company
NaCl	969.033.100	Isolab, Turkey
Glycerol	927.023	Isolab, Turkey
Trizma Base	T1503	Sigma-Aldrich, Germany
Agarose	HS-8000	Prona, Spain
Ethanol	920.026.2500	Isolab, Germany
Light Cycler 480 SYBR Green I	4887352001	Roche, Switzerland

Master		
QIAzol Lysis Reagent	79306	Qiagen, Germany
2-Propanol	961.023.2500	Isolab, Germany
Nonidet™ P40 (NP-40) Substitute	74385	Sigma-Aldrich, Germany
Dodecyl Sulfate Sodium Salt (SDS)	822050	EMD Millipore, USA
Pierce™ BCA Protein Assay Kit	23227	Thermo Fisher Scientific, USA
2-Mercaptoethanol	M3148	Sigma-Aldrich, Germany
N,N,N',N'- Tetramethyl ethylendiamine (TEMED)	110732	EMD Millipore, USA
Ammonium Persulfate (APS)	420627	AppliChem, USA
4x Laemmli Sample Buffer	1610747	Bio-Rad, USA
Methanol	947.046.2500	Isolab, Germany
Ponceu-S Solution	P-7170	Sigma-Aldrich, Germany
Albumin Bovine Modified Cohn Fraction (BSA)	SE1194301	Serva, Germany
TGX Stain Free FastCast™ Acrylamide Kit 10%	1610183	Bio-Rad, USA
Ethidium Bromide (EtBr)	17898	Thermo Fisher Scientific, USA

Tween-20	39796.51	Serva, Germany
Clarity Western ECL Substrate	1705061	Bio-Rad, USA
OneScript® Plus cDNA Synthesis Kit	G236	ABM, USA
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	M6494	Invitrogen, ThermoFisher
Methyl Cellulose	M-0387	Sigma-Aldrich, Germany

2.1.2 Cell Culture Products

The reagents used in cell culture experiments, their catalog numbers, and company information are listed in Table 2.2 below. Cell lines and primary cells used in the in vitro cell culture studies were MGG119-GFP. MGG119-GFP cells were obtained from Dr. Tuğba Bağcı Önder (Koç University).

Table 2.2 1 Cell culture reagents used for the in vitro experiments.

Product Name	Catalog Number	Company
Trypsin-EDTA Solution, 10X	B.X0930-100	Biowest, France
L-Glutamine Solution	B.X0550-100	Biowest, France
Sodium Pyruvate Solution 100mM	BI03-042-1B	Biological Industries, USA
Dulbecco's Modified Eagle Medium (DMEM) High Glucose,with L-Glutamine	BI01-052-1A	Biological Industries, USA
Penicillin-Streptomycin Solution	BI03-031-1B	Biological Industries, USA
Dulbecco's Modified Eagle Medium (DMEM) High Glucose, without phenol red	BI01-053-1A	Biological Industries, USA
Dulbecco's Phosphate Buffered Saline (D-PBS), 10X	BDL-002	Serena Europe, Australia
Sterile-filtered. Dnase/Rnase/Protease free water	WAT333.1L	Bioshop Canada, Canada
Dimethyl Sulfoxide (DMSO)	A3672	AppliChem, Germany

2.1.3 Solutions and Buffers

2.1.3.1 E3 Media

1X working solution E3 media were freshly prepared biweekly for raising zebrafish after fertilization until they were taken into the zebrafish tank system. 1X working solution was diluted from a 60X stock solution of E3. 60X stock solution was prepared by the addition of 17.2 g of NaCl (Sigma-Aldrich, 13423), 0.76 g of KCl (Sigma-Aldrich, 12636), 2.9 g of CaCl2.2H2O (Carlo Erba, 327607) and 4.9 g of MgSO4.7H2O (Sigma-Aldrich, M2773-1kg) in 1 liter of Milli-Q water. After mixing thoroughly, the solution was autoclaved and stored at 4°C. After diluting the 60X stock solution to 1X, 3-4 drops of 0.01% Methylene Blue were added to 1 L solution as an antifungal reagent.

2.1.3.2 Tricaine

A stock solution of 4 mg/ml (pH = 7.5) was prepared by mixing 400 mg of Tricaine with 2 ml of 1 M Tris (pH: 9.0). Then, pH was adjusted to 7.5 with HCl. The solution was filled with 100 ml of distilled water and stored at 4°C.

2.1.3.3 3% Methyl Cellulose

100 ml 1X E3 media was heated up to 60°C, and 3 grams of methyl cellulose powder was added and mixed until it dissolved. The solution was placed at -20°C and stirred every 30 minutes until

frozen. After overnight incubation at 4°C, it was aliquoted and stored in a zebrafish larval incubator at 28°C.

2.1.4 Laboratory Equipment

Laboratory equipment and devices used to carry out the experiments are listed below in Table 2.3 with their company information and their use of purpose.

Table 2.3 1 Laboratory equipment used in the experiments

Equipment Name	Company	Purpose
NanoDrop ND-1000	Thermo Fisher Scientific, USA	DNA & RNA quantification
PCR Thermal Cycler 2720	Applied Biosystems, USA	PCR & cDNA synthesis
LightCycler 96 Instrument	Roche, Switzerland	qPCR
Synergy HT Microplate Reader	BioTek, USA	BCA & MTT assays
DIC Microscope, DMi8	Leica, Germany	Imaging of cells
MN 120 Microbiological Safety Cabinet	Nüve, Turkey	Cell culture
Nuaire Autoflow IR Direct Heat CO2	Nuaire, UK	Cell Culture

incubator		
Themolyne Dri-Bath Heater	Thermolyne, USA	DNA extraction & Protein sample preparation
Universal 320 R Centrifuge	Hettich, Germany	Cell passaging
Nuve NB20 Water Bath	Nüve, Turkey	Cell culture
FemtoJet Express	Eppendorf, Germany	Xenograft
Narishige MN-151	Narishige, Japan	Xenograft

2.2 Methods

2.2.1 In vitro Methods

2.2.1.1 Thawing cells

Long-term cell stocks are kept in liquid nitrogen in cryovials, while short-term ones are stored at -80°C. After removing the cryovial, cells are thawed in a 37°C water bath until 90% of the tube content is thawed. The rest are thawed by mixing the cells in complete DMEM (Table 2.2). Cells are then transferred into 15 ml falcon in 5 ml complete DMEM to remove the freezing media and centrifuged for 5 minutes at 1500 rpm. After resuspending the cell pellet in 5 ml fresh complete DMEM, they were seeded into a T25 flask and kept in an incubator set to 37°C and 5% CO2. They can then be passaged into a T75 flask or 10 mm dish when they reach 70-80% confluency.

2.2.1.2 Passaging cells

The medium is aspirated from the flasks without detaching them, and wells are washed with pre-warmed 1X PBS (Table 2.2). Then, cells are incubated with 1X Trypsin-EDTA (Table 2.2) for 3 minutes to detach them. After they are detached, complete DMEM that is at least two times the amount of Trypsin-EDTA was added to inactivate the effect of the trypsin, and cells are transferred into a 15 ml falcon. The tube is centrifuged for 5-7 min. At 130g, the pellet is dissolved in complete fresh media. Depending on the growth rate of the cells and the size of the flask which cells are transferred into, cells are split into multiple flasks. For the cell lines used in our research, A172 and U87MG, 1:2 to 1:5 subcultivation ratios are applied.

2.2.1.3 Freezing cells

To maintain healthy stocks, cells are frozen in their early passage states, such as in their second or third passages. After trypsinizing the cells, complete fresh media is added, and cells are centrifuged for 5 minutes at 1500 rpm, as mentioned in the "Passaging cells" section. A freezing medium is prepared.

2.2.2 In vivo Methods

2.2.2.1 *Danio rerio* Xenografts

Xenografts were performed using the Femtojet Express microinjector (Eppendorf, Germany) and Narishige MN-151 manipulator (Narishige, Japan). 1.0 mm x 0.5 mm x 10 cm sized borosilicate

capillaries (Sutter Instrument, USA) were loaded with 5 ul of cells at a time and injected manually into the animal pole of the blastula stage zebrafish and yolk of 2 dpf zebrafish. 5 nl of cell volume was injected in one press for every embryo.

2.2.2.2 Stereo-fluomicroscope Imaging of *Danio rerio* and Statistical Analysis of Images using ImageJ

All zebrafish were anesthetized with Tricaine (4 mg/ml) before imaging. 2-3 drops of Tricaine were added into a plate holding approximately 30 ml E3 media and waited until the zebrafish stopped swimming before imaging. At 5 dpf, all zebrafish were imaged before finalizing the experiment. Stereo-fluo images of the zebrafish larvae at 3.2X and 8X objectives were taken to get a full lateral view and a more focused brain/yolk image. Larvae were positioned onto the slides, laying on their side with a slight drop of water to get the best image quality. Brightfield and fluorescent images at both objectives were taken for each sample without moving the fish. All the analyses were performed using the ImageJ application. From the measurement tab, Area, Mean, IntDen, and RawIntDen were selected to calculate CTCF (corrected total cell fluorescence). Since it measures corrected fluorescence, the background signal needs to be removed. Thus, 3 random areas from the background (with no signal) were selected, and the mean and area for each of them were calculated. Later, regions with the GFP signal were hand-selected for each fluo-image, and the above attributes were calculated. The background mean was calculated by taking the average of 3 background areas. Finally, for each fish, the measured signal area was multiplied by the background mean, and then the value was removed from the IntDen of each fluo-image to calculate CTCF. For the gall blader analysis, XM, YM, and StdDev

parameters were also selected from the measurement tab. Similarly, background and signal areas were hand-selected, and selected parameters were measured for each larva. XM and YM measurements provide the center of mass information about the signal area and how dispersed the signal is laterally and vertically from the center. StdDev allows Z-scores to be calculated as the Mean is also measured. By plotting the Z-score graph of each group and comparing them with one another, how each group's signal is distributed can be observed. Moreover, merged images of brightfield and fluorescent images can be created by Image J to better localize the signal on the brightfield image. To a merged channel image, both brightfield and fluo images were first loaded and turned to 8-bit type. Then, from Process they were sharpened. Finally, using Merge Channels, each image type's channel (i.e., bf or fluo) was selected, and a merged image was created. Statistical analysis of the measured parameters was performed using GraphPad Prism. To compare all groups in terms of their CTCF, IntDen, Area, StdDev, and Z-scores, ordinary one-way ANOVA with Tukey's multiple comparisons with 95% CI was performed. For the center of mass analysis, since two parameters' (XM, YM) interaction between groups was trying to be visualized, two-way ANOVA with Tukey's multiple comparisons with 95% CI was performed.

2.2.3 The Curation of Biological Data

The expansion and generation rate of biological research has increased the production of biological data that needs to be classified, categorized, and analyzed over the past few years [79]. The curation of biological information is a critical method as it enables the reviewing, verifying, and organizing of different research into a compact form. This process increases data

accessibility, promotes reproducibility, and facilitates knowledge synthesis, leading to a faster understanding of ever-evolving biological data [80]. Professional curators are often Ph.D.-level experts in their area who annotate critical information and update content as the literature expands to organize and hold biological data in a semantically standardized way [81]. For example, platforms such as UniProt and PDB require expert curators to organize their knowledgebases using unique and traceable identifiers [82], [83]. Aside from the molecular level, information about a whole organism can also be curated. Model organisms like *Drosophila melanogaster, Schizosaccharomyces pombe, and Danio rerio* have their own online databases formed by professional curators such as FlyBase, PomBase, and ZFIN, respectively [84]–[86].

Chapter 3

Results

3.1 Overview of ZenoFishDb Glioma Database

ZenofishDb Glioma database is a Streamlit application created with Python. It holds information about zebrafish glioma transplantation studies in a user-friendly interface, allowing users to review the most updated literature and filter necessary information. It is an improved, more detailed, and focused version of its predecessor ZenofishDb v1.1, https://konulab.shinyapps.io/zenofishdb/. Within the current version of ZenofishDb Glioma, detailed information about glioma transplantation studies in terms of the cell lines, PDX (patient-derived xenograft) models, zebrafish lines, molecular modifications of the cells, injection and post-injection assessment times, and more can be found as highlighted in the homepage (Figure 3.1). In the process of collection of these data, PubMed was searched with the "zebrafish glioma xenograft," "zebrafish glioblastoma xenograft," "zebrafish glioma transplantation," and "zebrafish glioblastoma transplantation" keywords and all publicly available relevant research until February 2022 were harbored into the database. Apart from the data collection of the characteristics mentioned above of the publications, ZenofishDb Glioma also provides the

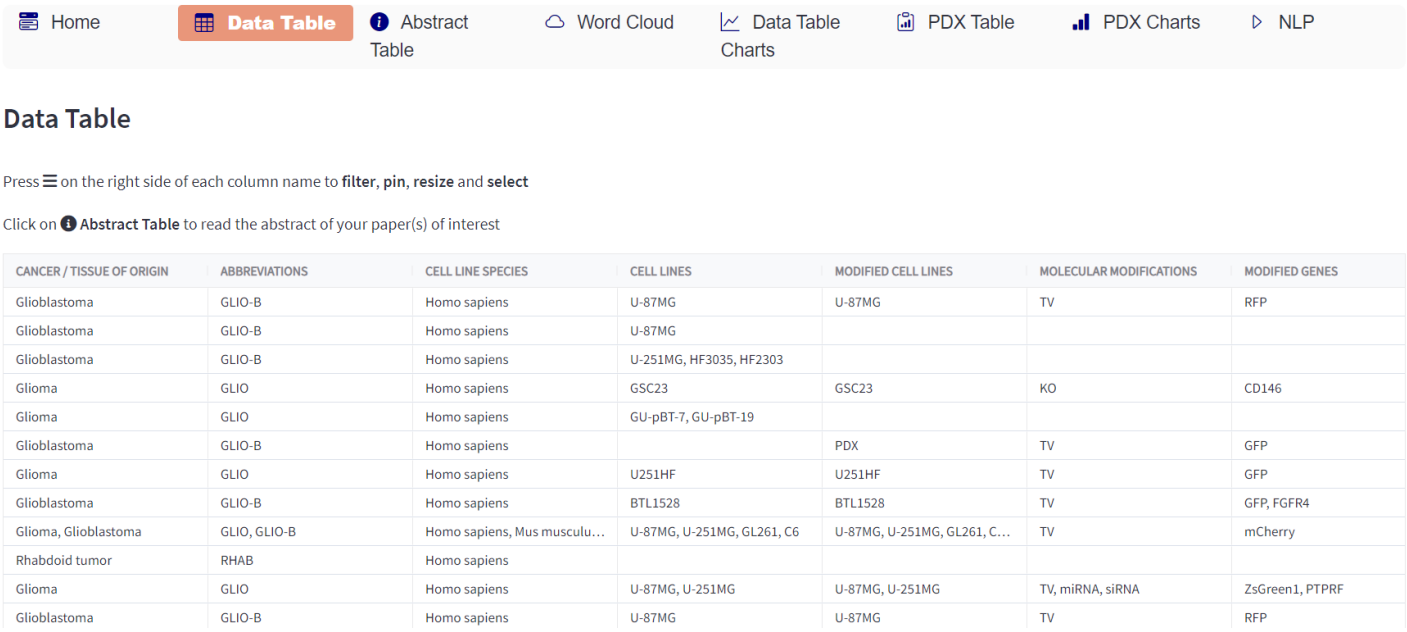
abstracts and text information about the zebrafish xenograft part of the study with its corresponding figures with the help of text mining and natural language processing (NLP). Moreover, a word cloud based on abstract information can be visualized to see the keywords of each research without going into a deeper dive.

Table 3.1 1 Comparison between ZenofishDb Glioma and ZenofishDv v1

ZenofishDb Glioma	ZenofishDb v1
Manual curation of 42 papers	Manual curation of 200+ papers
Includes zebrafish glioma xenograft studies only	Includes zebrafish xenograft studies
Data visualization of manual curations by bar charts	Data visualization of manual curations by bar charts and pie charts
Provides papers' abstract and title information of the	-
Creates a wordcloud based on abstracts of the papers	-
Gives summary of the abstracts of the papers	-
Provides the zebrafish-related experiment figures of the open access papers	-
Gives summary of the full-text of the open access papers	-

Figure 3.1 1 ZenoFishDb Glioma Homepage. The welcome page of the app highlights its purpose and function. At the top panel, Home, Data Table, Summary Table, Word Cloud, Visualization, PDX Details, PDX Charts, and NLP tabs are provided. At the bottom of the page, templates for data curation can be downloaded for user submission.

3.2 Structure of the ZenoFishDb Glioma Database



CANCER / TISSUE OF ORIGIN	ABBREVIATIONS	CELL LINE SPECIES	CELL LINES	MODIFIED CELL LINES	MOLECULAR MODIFICATIONS	MODIFIED GENES
Glioblastoma	GLIO-B	Homo sapiens	U-87MG	U-87MG	TV	RFP
Glioblastoma	GLIO-B	Homo sapiens	U-87MG			
Glioblastoma	GLIO-B	Homo sapiens	U-251MG, HF3035, HF2303			
Glioma	GLIO	Homo sapiens	GSC23	GSC23	KO	CD146
Glioma	GLIO	Homo sapiens	GU-pBT-7, GU-pBT-19			
Glioblastoma	GLIO-B	Homo sapiens		PDX	TV	GFP
Glioma	GLIO	Homo sapiens	U251HF	U251HF	TV	GFP
Glioblastoma	GLIO-B	Homo sapiens	BTL1528	BTL1528	TV	GFP, FGFR4
Glioma, Glioblastoma	GLIO, GLIO-B	Homo sapiens, Mus musculu…	U-87MG, U-251MG, GL261, C6	U-87MG, U-251MG, GL261, C…	TV	mCherry
Rhabdoid tumor	RHAB	Homo sapiens				
Glioma	GLIO	Homo sapiens	U-87MG, U-251MG	U-87MG, U-251MG	TV, miRNA, siRNA	ZsGreen1, PTPRF
Glioblastoma	GLIO-B	Homo sapiens	U-87MG	U-87MG	TV	RFP

Figure 3.2 1 ZenoFishDb Glioma Data Table tab. Data Table tab classifies information about glioblastoma zebrafish xenograft studies based on cell lines, molecular modifications, zebrafish lines, and study type.

The Data Table is comprised of 42 manually created glioma zebrafish xenograft studies based on 23 attributes such as cell lines, molecular modification, modified genes, zebrafish line, location of the injection, study type, etc. The data table enables automated filtering of the wanted search and enables users to refer to the PMID of the interested paper (Figure 3.2).

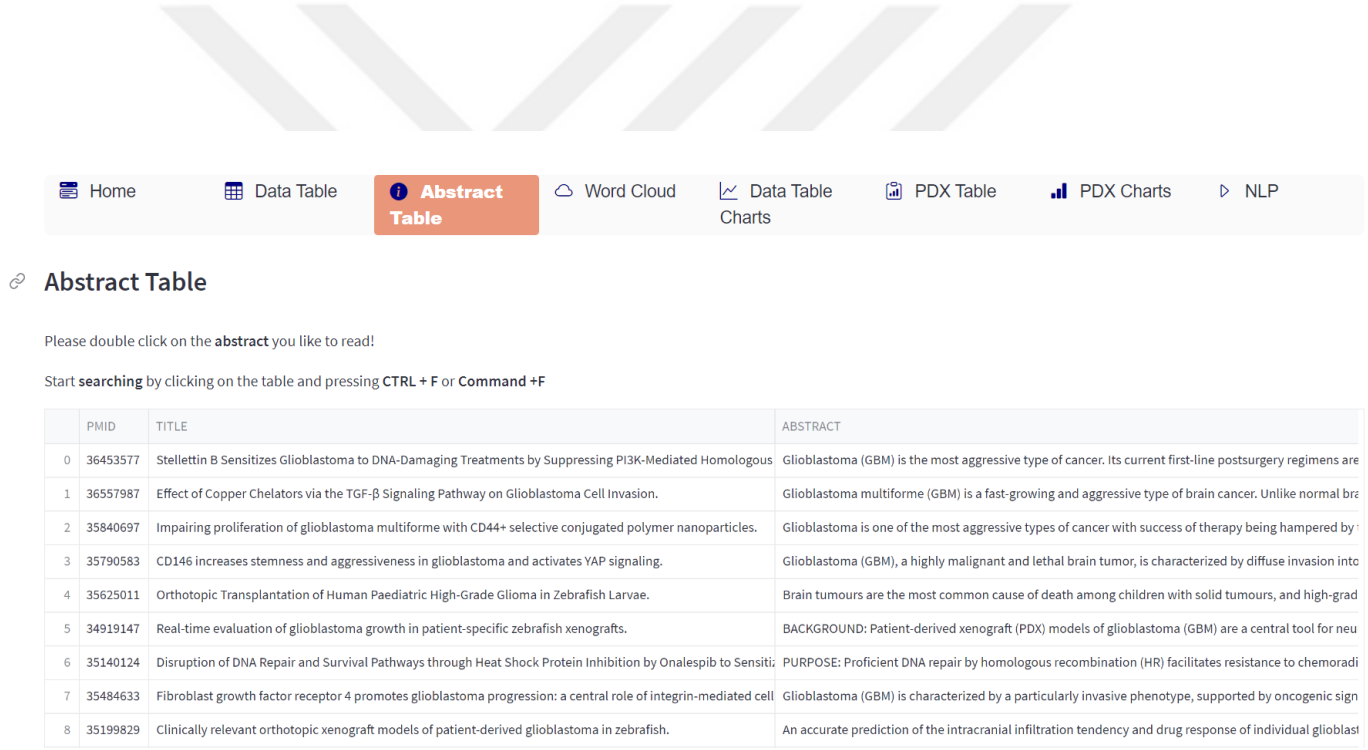


Figure 3.3 1 ZenoFishDb Glioma Abstract Table tab. Abstract Table tab holds a repository of titles and abstracts of the zebrafish glioma xenograft studies for easy access.

Upon interested PMID(s) is retrieved, users can move to the next tab, Abstract Table, to read more in-depth about the paper they are interested in. This tab helps users decide whether the paper they initially chose is what they are looking for (Figure 3.3). After reading the abstract, they can also visualize its word cloud and read about the abstract's summary (Figure 3.4).

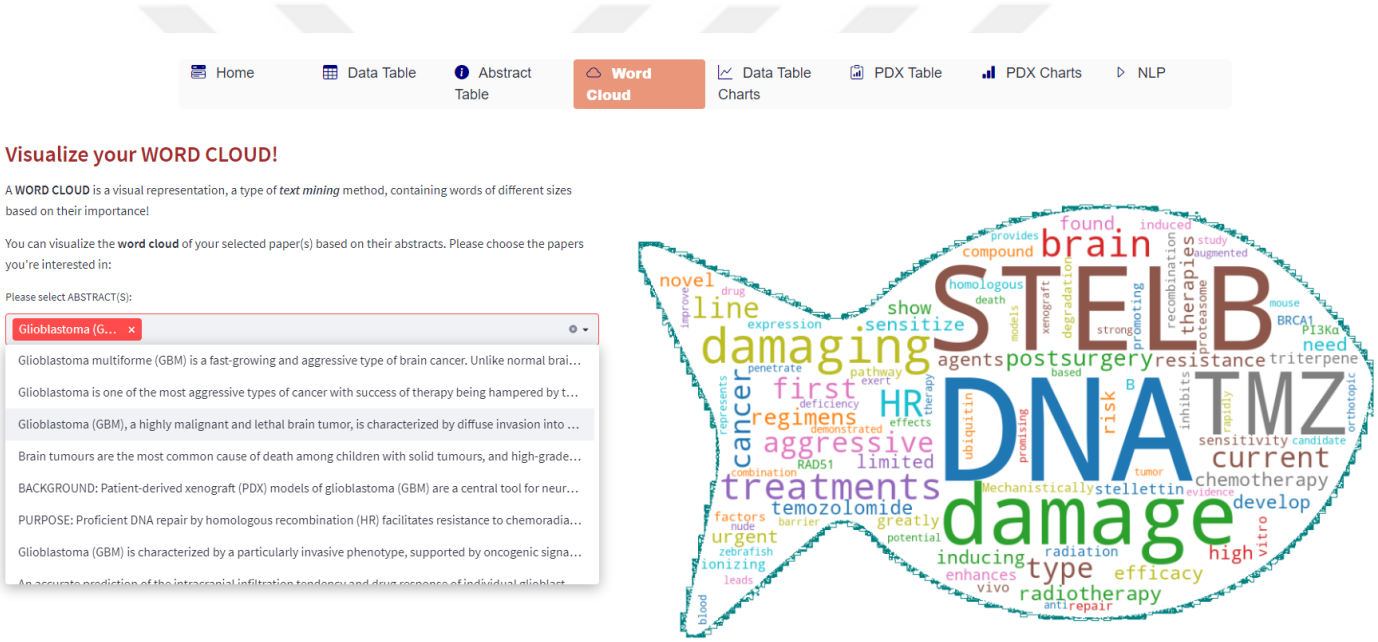


Figure 3.4 1 ZenoFishDb Glioma Word Cloud tab. This tab allows users to pick an article(s) from the dropdown menu on the left and visualize its abstract's word cloud on the right.

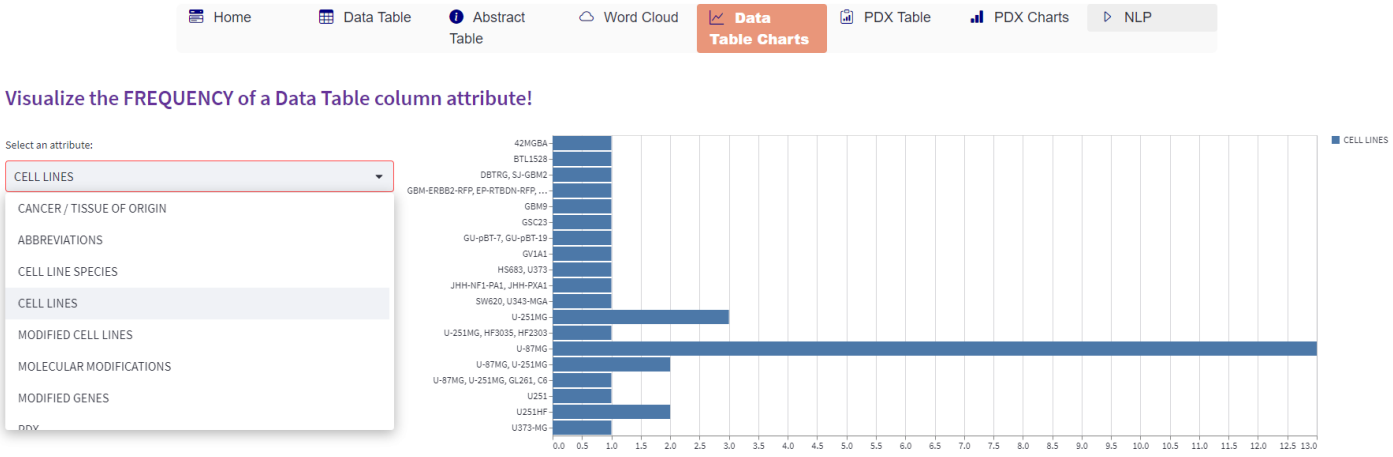


Figure 3.5 1 ZenoFishDb Glioma Data Table Charts tab. Data Table Charts tab allows users to select a Data Table column to visualize the frequency selected column.

Data Table Charts and PDX Charts offer frequency information about the repository in the shape of a bar chart using the altair package. Users can choose the characteristic they are interested in or want to learn more about by choosing from the dropdown menu (Figure 3.5, Figure 3.7).

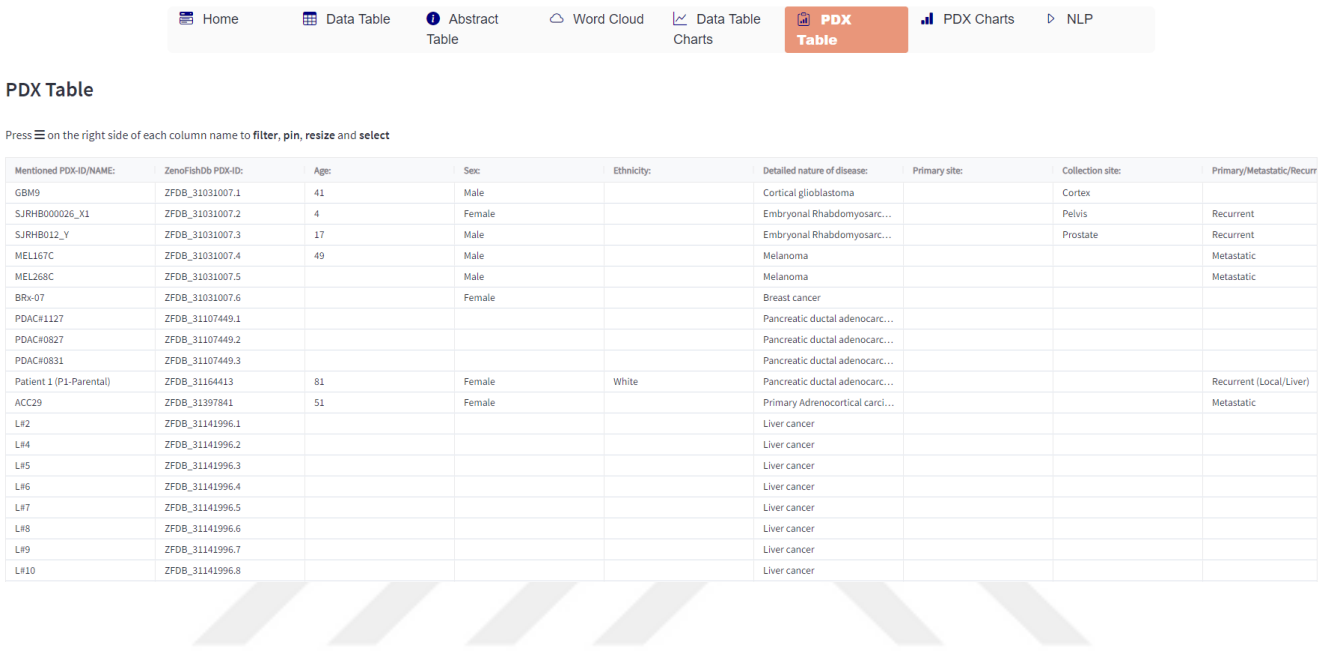


Figure 3.6 1 ZenoFishDb Glioma PDX Table tab. The PDX Table tab classifies PDX information about glioblastoma zebrafish xenograft studies based on age, sex, ethnicity of the patients and collection site, tumor mutation properties, and more.

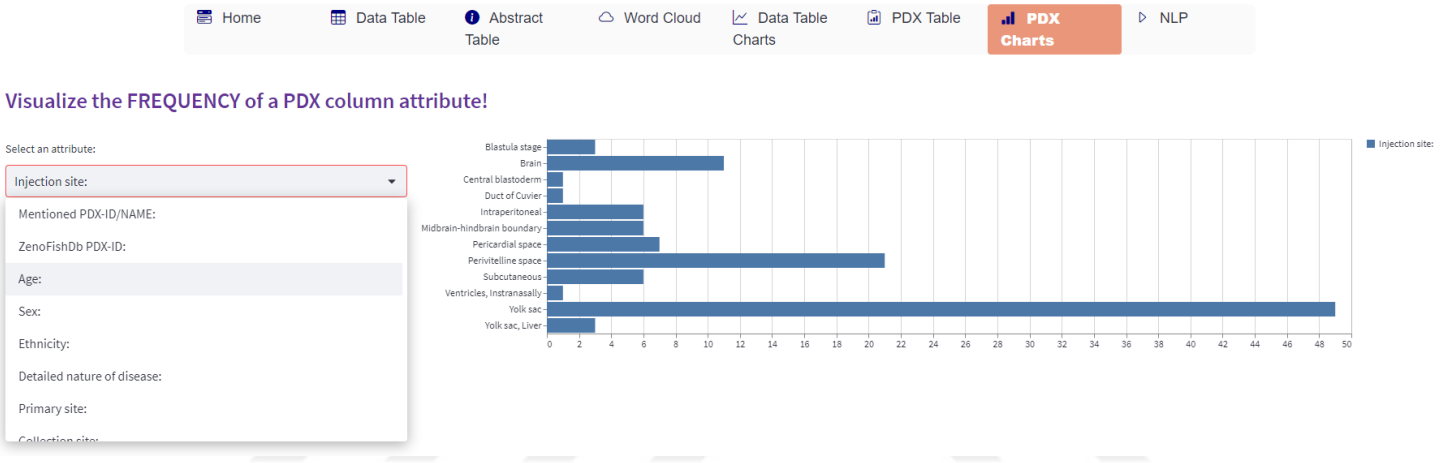


Figure 3.7 1 ZenoFishDb Glioma PDX Charts tab. The PDX Charts tab allows users to select a PDX Details column to visualize the frequency of the selected column.

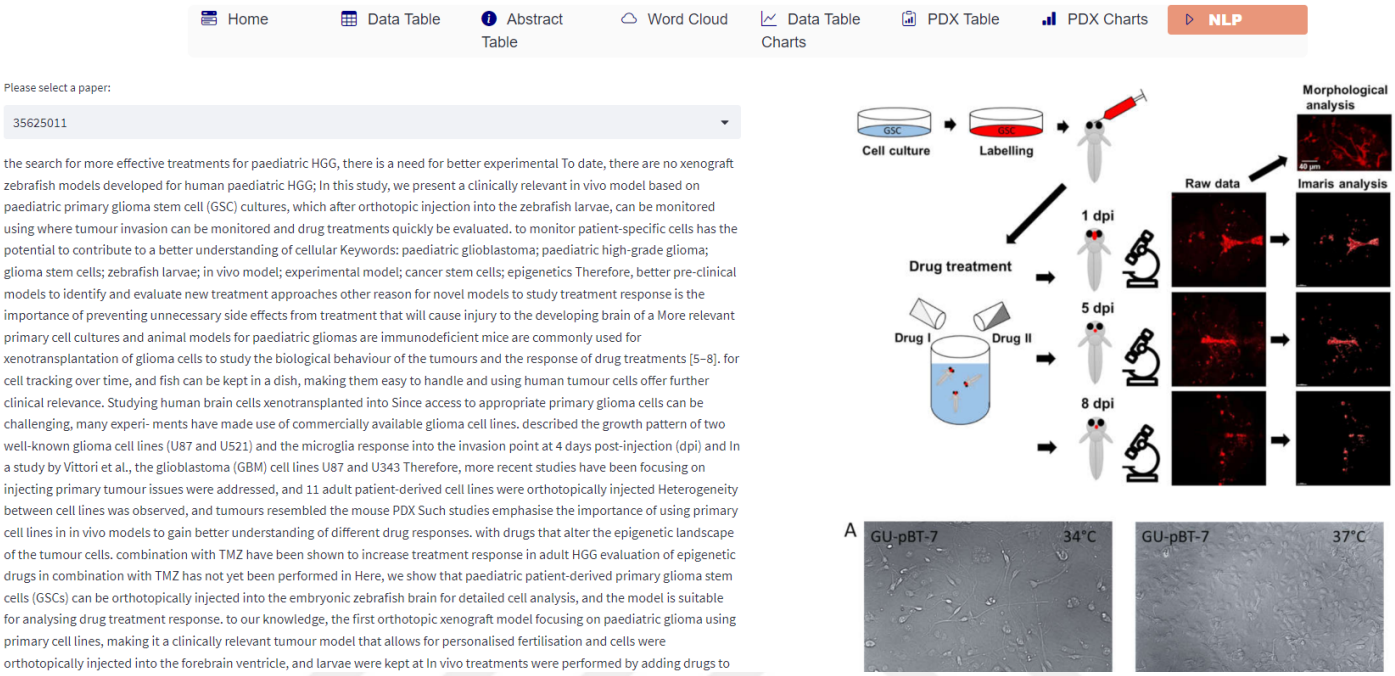


Figure 3.8 1 ZenoFishDb Glioma NLP tab. The NLP tab allows users to select a paper from the dropdown menu on the left. After selection, it allows users to read the "zebrafish xenograft" related part of the paper with its related figures. Also, upon selection, it provides a summary of the "zebrafish xenograft" related part.

The NLP tab provides open-access data of glioma zebrafish xenograft studies with their respective figures and summaries. pdfminer and gensim libraries were used to extract Pubmed papers and summarize "zebrafish-related parts". This tab allows users to both read and take a look at the figures in a less time-consuming manner (Figure 3.8).

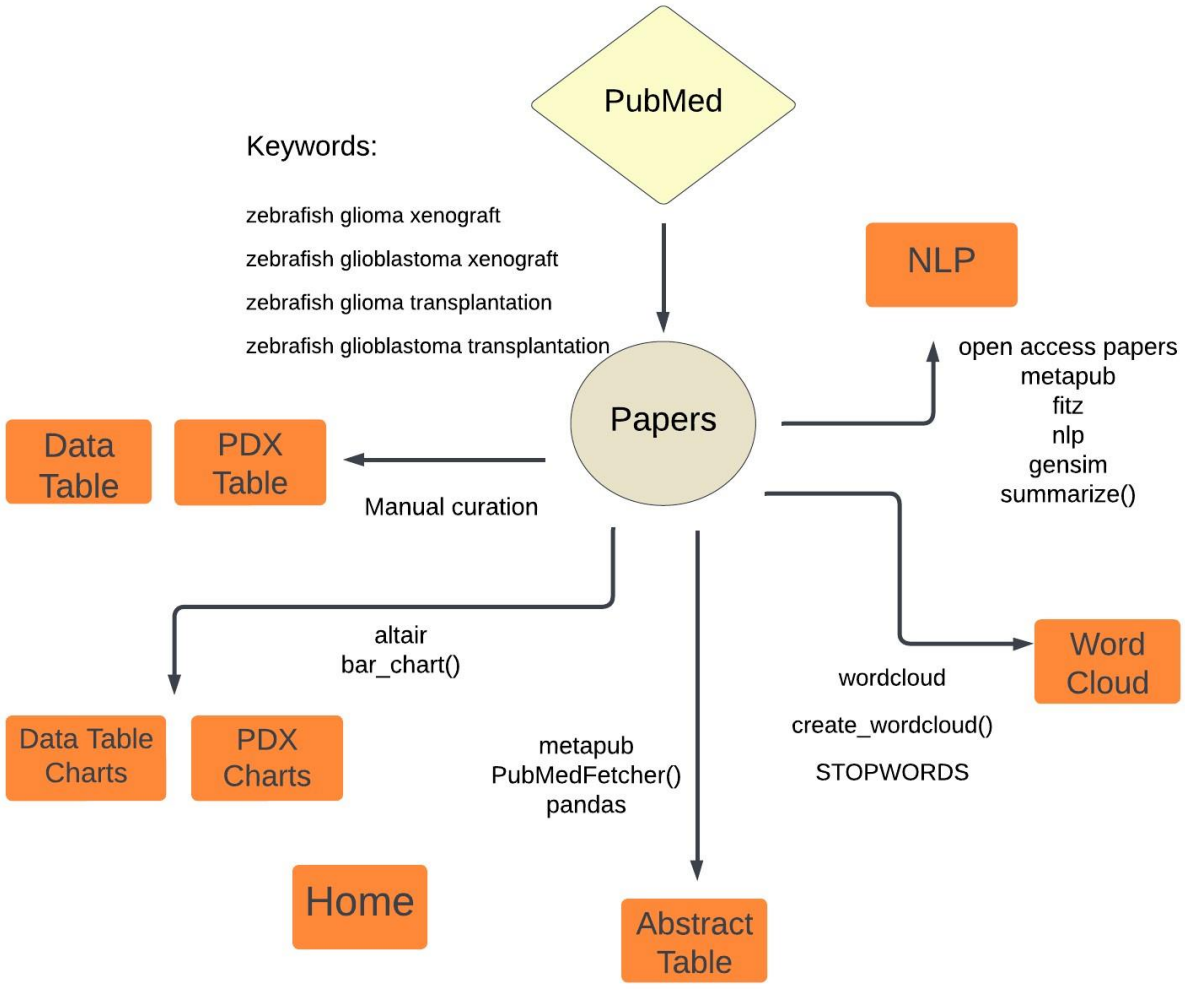


Figure 3.9 1 Diagram of the ZenofishDb Glioma application showing key libraries and functions used in the development of each tab.

Before the beginning of the development of the app, manually curated excel files of Data Table and PDX Table were prepared for 42 zebrafish glioma xenograft studies searched on PubMed.

Then, using the PMIDs of the selected papers, the abstract and title data of the papers were retrieved and stored in an excel file. During this Process, the metapub library was used to create a Pubmed object with the function PubMedFetcher(), called fetch. Using fetch with the functions article_by_pmid.abstract and article_by_pmid.title, abstract and title information for each PMID was obtained. Obtained PubMed information was merged with the help of pandas library and written as .csv to be uploaded to the Abstract Table tab of the app.

For preparing the word cloud tab, retrieved abstract information was used. A zebrafish-shaped visualization method was created using the word cloud library and create_wordcloud() function. Since a word cloud decides whether a word is significant in a given context based on its repetition and small words such as it, a, an, and along with keywords such as glioma, glioblastoma, cell, cells, GBM were eliminated using STOPWORDS. Upon a selection of a specific paper(s) from the dropdown menu, a word cloud of the selected abstract(s) can be visualized, and a summary of the abstract(s) can be read as well.

Data Table Charts and PDX Charts tabs were coded using the altair library with table_barchart() function to visualize the frequency of each category of the respective manually curated files.

For the development of the NLP tab, only open-access papers were used. Firstly, using the metapub library and urlretrieve() function, PMIDs of the open access papers, which are held in a list, were looped through to retrieve the selected papers' URLs on a temporary disk location. Then, with the help of the fitz library, those URLs were opened in the form of .pdf and stored in a file. Finally, using the NLP library, collected PDFs were looped to extract images with the

extract_image() function. Extracted figures of each paper were collected with their original extensions (.png, .jpg, etc.) so as not to create any deformations while retrieving, separately for each PMID. To create one image file for each open-access paper, collected figures related to zebrafish glioma xenograft experiments were manually merged using Figma. Upon the selection of an open-access paper from the dropdown menu, the NLP tab provides the respective figures of the paper with a full-text summary of the study. Summarization of abstracts in the Word Cloud tab and summarizing full texts in the NLP tab were created with the help of the genism library and summarized () function. The ratio parameter of this function ranges between 0 and 1 and decides the proportion of the number of sentences of the original text to be chosen for the summary. So, I set it to 1 to get a full-text summary. Another parameter, word_count, determines the word limit of the summary. Since when both parameters are set, the ratio will be ignored; I didn't set a value for the output limit. Thus, using this function, I could get a non-biased and random summary of the selected study.

Table 3.2 1 Frequencies of most commonly researched attributes of zebrafish glioma xenograft model extracted from ZenofishDb Glioma app

Attribute	Most commonly researched	Study #
Cell line	U87-MG	16
Molecular modification	TV	27
Modified gene	GFP	13
Treatment	Temozolomide	9
Injection site	Yolk sac	15
Developmental stage	Embryo	41
Injected cell numbers (categorized)	50<n<=200	23
Injection time	48 hpf	25
Tumor assessment time	72 hpi	8
Zebrafish line	Wild type	16
Cell tracking source	GFP	14
Analysis	Tumor growth	19

3.3 MGG119-GFP primary glioblastoma cell line injection into Casper zebrafish for the optimization of a xenograft protocol

Development and curation of ZenofishDb Glioma application have shown that there are different ways to transplant cells or PDX tissue in zebrafish based on the cell line and the age of the

zebrafish. That is why it is crucial to optimize the injection method before moving on to more advanced analysis after injection.

MGG119 primary cell line (kindly gifted by Dr. Tugba Bagci, Koc University) was used to set a xenograft protocol in larval zebrafish to study tumor growth, metastasis, and possible drug therapies later. After the embryo collection, one group of Casper embryos were raised inside an incubator at 28°C until they reached 2 days post fertilization, while another group was utilized in a blastula injection 3-4 hours after they were born. On the day of transplantation for each group, early in the morning, MGG119-GFP was prepared for the injection by harvesting and counting at different numbers to find the optimized injection amount. The number of cells harvested was adjusted by the injection volume per embryo, which was set to 5 nl. Keeping transplanted cell numbers uniform for each embryo is critical to later measuring tumor invasion, metastasis, or burden. Then, injection time was automatically set, and a 5 nl volume was injected into each embryo. After a literature search and considering the shape and growth pattern of MGG119-GFP cells, 50, 100, 200, and 300 cells per embryo were selected as different injection groups. Since they were already tagged with tracker protein - GFP, usual dyeing protocols of the cells with DiI or DiO were not applied.

After the preparation of the cells, 2 dpf embryos were dechorionized manually using tweezers with minimum force on the animal, whereas embryos that were harbored in blastula injection were not manipulated. Embryos were lined up on the side of a slide inside a petri dish with a minimum amount of E3 to prevent them from drying during blastula injection. On the other hand, during 2 dpf injection, the first larvae were anesthetized with Tricaine to prevent movement. Then, they were lined up in the wells of a mold to immobilize them during injection. At least 30

embryos for each group were injected and kept in a 33.5°C incubator till they reached 5 dpf. They were checked regularly (Figure 3.10).

After the imaging, results showed that there was indeed a significant difference between the smallest injection amount – blastula 50 cells injection and other groups in terms of both CTCF and intensity density, which are two different methods that measure signal amount after injection. Yolk injection with 200 cells showed the highest signal density among the three, slightly more elevated than blastula injection with 300 cells. Although there was no significant difference between the two findings, further wet lab search can help differentiate the importance of the location and amount of cells injected. Findings also showed a larger area on injection was observed with more cells injected. Moreover, there was a significant difference between the blastula 50 group and blastula 300 and yolk 200 groups, respectively, although there wasn't any between the two (Figure 3.11). As seen in Figure 3.10, a visible gall bladder signal was observed in every experiment group. That's why I also analyzed the gall bladder region but couldn't find a significant difference in signal density comparing Z-scores. Considering the high standard deviation range between and within the groups, it can be said that it is considered an effecting factor. The only significance was observed between the groups' blastula 300 and yolk 200 injections; yet again, the standard deviation effect should not be forgotten.

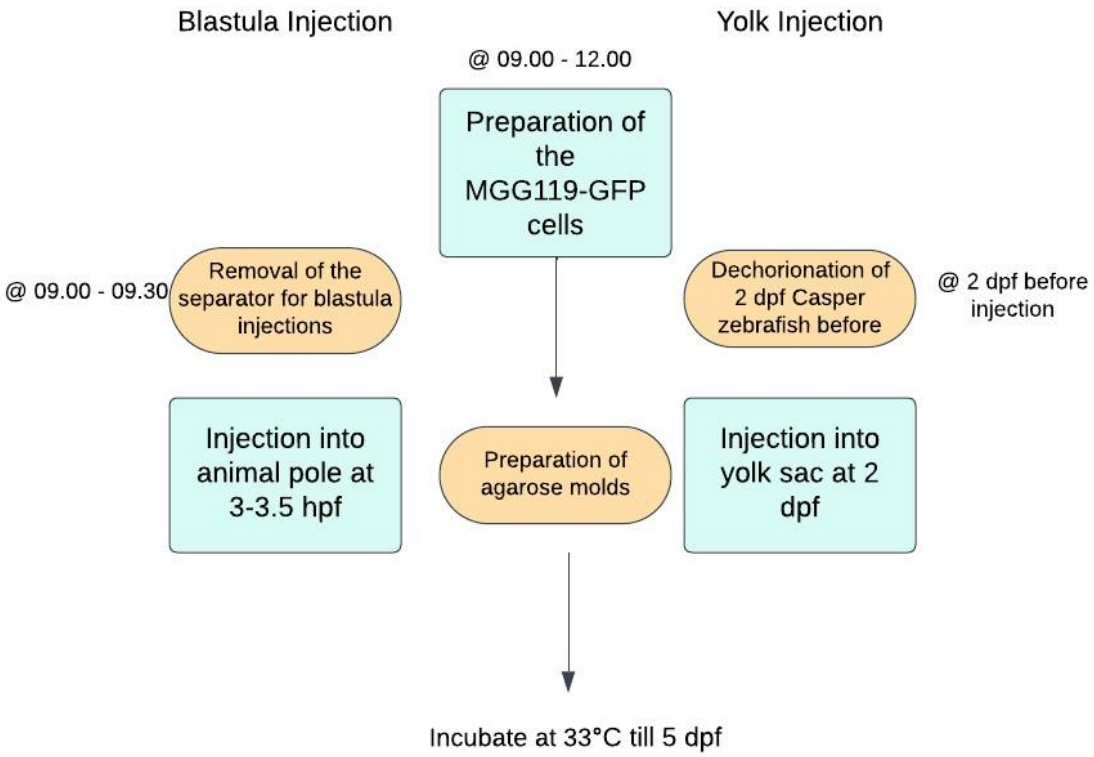


Figure 3.10 1 Diagram of experimental plans for the preparation and implementation of zebrafish glioma xenograft.

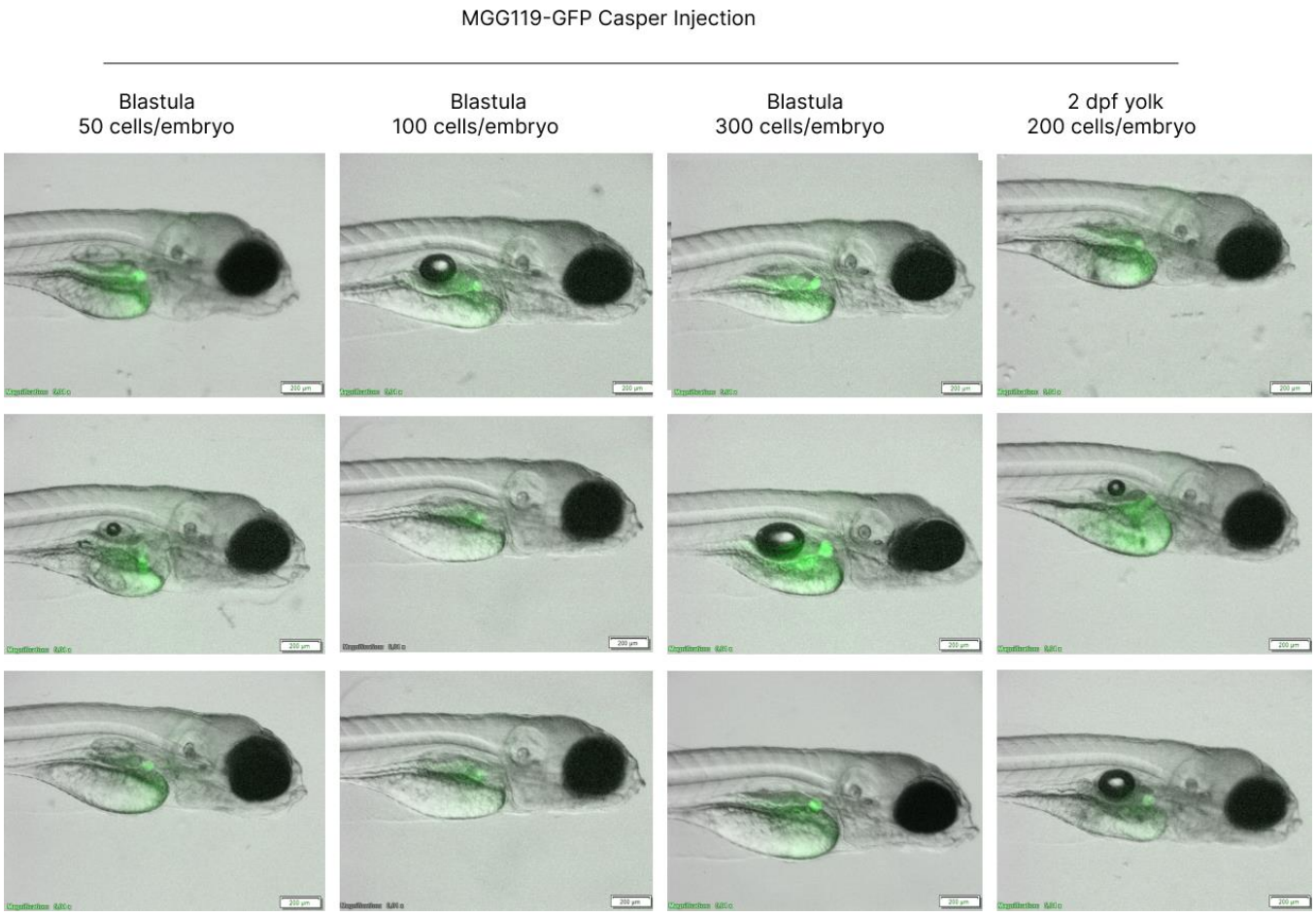


Figure 3.11 1 Merged 5 dpf stereo-fluorescent images of MGG119-GFP cells injected into Casper blastula and 2 dpf for cell injection optimization. Groups: 50 cells/embryo, 100 cells/embryo, 300 cells/embryo (blastula), and 200 cells/yolk (2 dpf). Three representative figures are put for each group. Scale bar = 200 uM.

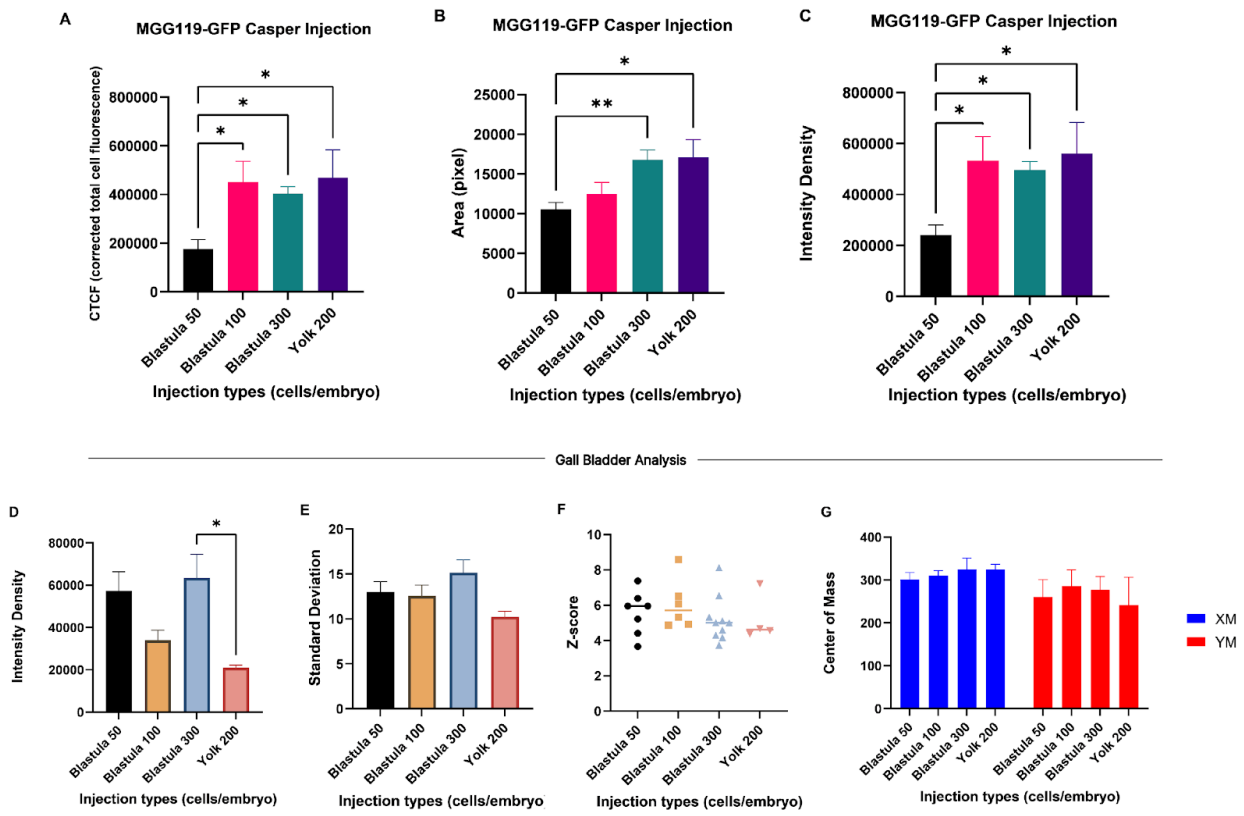


Figure 3.12 1 Merged 5 dpf stereo-fluorescent image analysis of MGG119-GFP injected Casper embryos at blastula stage and 2 dpf by Image J. Top panel: Signal quantification comparison between all groups. Corrected total cell fluorescence (A), signal area (B) and intensity density (C). Bottom panel: Gall bladder signal quantification comparison between all groups. Intensity density (D), standard deviation (E), Z-score (F) and center of mass (G). p value<0.05 indicates *, p value <0.01 indicates ** One-way ANOVA with Tukey's multiple comparisons (A-F). two-way ANOVA with Tukey's multiple comparisons (G).

Chapter 4

Conclusions and Discussion

4.1 Conclusions

In the most updated and current version, which was presented in this thesis, the ZenofishDb Glioma application holds information about zebrafish glioma xenograft studies by categorizing them based on the characteristics such as cell line used, molecular modifications, zebrafish lines, PDX tissues used, and the type of injection. This application is not only manually curated but also provides text mining of related research using natural language processing, which builds on its predecessor as it only utilizes the power of manual curation. It also provides a more focused repository, offering only glioma studies of the zebrafish xenograft models. Using summarization methods of related text - glioma zebrafish xenograft - and extracting images of the interesting part of the research, this application provides a broad spectrum of information from abstracts to word clouds on top of manual curation.

Like ZenofishDb Glioma, there are multiple knowledgebase repositories for model organisms such as WormBase, FlyBase, Mouse Genome Database, Rat Genome Database, and Saccharomyces Genome Database. WormBase can offer information about *C. elegans* and nematode biology by allowing users to search and filter through different genes, antibodies, proteins, and phenotypes [87]. Like WormBase, Mouse Genome Database can offer search and filter functions and analysis tools to retrieve gene attributes and compare genome features of *de*

novo sequenced mouse strains [88]. Different from the mentioned, ZenofishDb Glioma uses the power of NLP to collect and present the related and current zebrafish glioma xenografts. Not only is PDX-Finder one of the biggest knowledgebase repositories, but it also holds information about 4974 PDX rodent models and 380 rodent organoid models [89]. Like the ZenofishDb Glioma PDX Table, PDX-Finder provides information about the tumor type, collection site, primary site, patient sex, and more [89]. To expand the ZenofishDb Glioma app's perspective on PDX data, PDX-Finder characteristics can be utilized. As one of the most studied animal models, the Mouse Models of Human Cancer Database acts as a xenotransplantation catalog offering PDX data, genetically engineered mouse models, inbred mouse strains, and mouse models mimicking human diseases [90]. Similar to MMHCdb, the ZenofishDb Glioma app also offers similar characteristics about zebrafish, such as strain information of the organism, tumor type, and human tissue/cell transplantation. Although zebrafish is considered a great model for cancer research due to its low cost-high efficiency ratio, compared to more common murine organisms, zebrafish-specific knowledgebase platforms are in need. ZFIN is the most comprehensive and updated repository, serving as a search and filtering tool for zebrafish-related genes, expression data, mutants, transgenic lines, and antibodies. It also provides basic tools like zebrafish specific-BLAST to align nucleotide and protein sequences [91]. Unfortunately, ZFIN doesn't offer any automated curation model using ML power as other mentioned repositories above. One promising application by Groenewoud et al. has been created to establish standardized protocols for zebrafish xenotransplantation and share xenograft phenotypes [92]. XePhIR currently holds 6 zebrafish xenograft studies, 1 breast cancer, and 5 osteosarcoma, and provides visual phenotype data and meta-information about the study, similar to ZenofishDb Glioma [92]. Moreover, it offers generalized protocols for cell preparation before injection, retro-orbital engraftment (RO),

perivitelline space engraftment (PVS), and hind brain cavity (HBC) engraftments [92]. Compared to ZenofishDb Glioma, XePhIR holds a lot fewer studies and doesn't provide comprehensive meta data and study figures; on the other hand, it offers protocols and ImageJ analysis methods which can also be incorporated into ZenofishDb Glioma in the future.

A comprehensive and recent review paper by Pliakopanau et al. shows similar characteristics as ZenofishDb Glioma in the sense that they were also summarizing advances in GBM xenografting through literature curation [93]. Although they didn't make an app from their findings, similar outcomes and assumptions can be seen between the ZenofishDb Glioma app and their review article. Similar to the biocuration process I performed with "zebrafish glioma xenograft," "zebrafish glioblastoma xenograft," "zebrafish glioma transplantation," and "zebrafish glioblastoma transplantation" keywords in PubMed, they searched ZFIN, PubMed and Scopus with certain keywords such as "glioblastoma," "xenotransplantation," and "zebrafish" [93]. After they collected all the papers, they eliminated duplicates, not-involving zebrafish, and not-involving xenotransplantation as I did in my filtering process. One main difference, however, can be seen in the search date. They preferred to restrict their curation from 2005 to 2022 (October 30), whereas, in ZenofishDb Glioma, all papers till March 2023 can be found [93]. Another point that differs is that they only focused on incorporating glioblastoma data, resulting in 46 total papers, while I searched for all types of gliomas resulting in 42 papers. They also created a curation table holding information about zebrafish strain, cancer cell line, cell labeling method, cell number, time of injection, injection site, and maintenance temperature [93]. Except for the maintenance temperature, all the parameters they included exist in the ZenofishDb Glioma curations, and even more can be found, such as analyses performed, developmental stage, host modification details, PDX data, treatment, tumor assessment time, stem cell properties,

molecular modifications. Looking at their results, it can be seen that our findings lead to shared outcomes. Their review designated that Wild type, Casper, and Tg(fli1:EGFP) zebrafish predominate among other strains, which can also be concluded from ZenofishDb Glioma [93]. App shows that the most commonly used strain is AB (16 papers), then Tg(fli1:EGFP) (9 papers), and finally Casper (7 papers). Another parallel finding is that the most effective injection is at 48 hpf with 50-100 cells/embryo [93]. It can also be seen in the app that the most commonly used injection time is 48 hpf, while the cell number injected ranges between 50-200. Unlike ZenofishDb Glioma, they also concluded that angiogenesis studies mainly use U87MG cells, and proliferation studies and PDX models mainly use U251MG cells [93]. Tumor growth, proliferation, angiogenesis, and migration are the mainly researched topics in the app as well, but since there isn't any 1-to-1 match and search between cell lines and analysis types, this can't be concluded from the app. So, not only does ZenofishDb Glioma bio curate entire zebrafish glioma xenografting literature, but it also provides a full-text summary, abstract summary, word cloud, and figure extraction by powering NLP methods, unlike other knowledgebases. This puts ZenofishDb Glioma as an NLP-based web tool with a comprehensive repository power in glioma xenotransplantation and offers a good prototype for scientists.

Natural language processing offers great opportunities not only for science and technology research but also in the health sector. Hasan et al. show the importance of NLP by implementing an NLP-based chatbot that uses computer vision in their app, allowing mental health seekers to register and use functionalities for personality assessment and chatting with like-minded people, especially during the COVID-19 era [94]. Another exciting research utilizing NLP develops a tool creating multimodal interaction on existing applications by adding voice command modality. By accepting the user's voice input, the tool detects intents by creating

language models and performing the requested information [95]. As telemedicine over the web is becoming more and more prominent nowadays, this smartphone-based app captures patient data to implement into different medical knowledge platforms and, with the help of AI, offers differential diagnoses over the phone depending on patients' need [96].

Upon the development of the ZenofishDb Glioma repository, data collected show that most glioma injections performed in zebrafish happened at 48 hpf [34], [48], [97]. Also, the most common research interest of glioma injections can be concluded as tumor formation and growth analysis, migration, and survival [98]–[100]. While most of these studies use similar cell lines such as U87MG, A172, U251MG, and U373 and similar zebrafish lines such as Wild type, Casper, or transgenic lines to visualize vasculature, they don't offer a uniformed methodology in terms of injection style. For example, Fan et al. injected 200 U87MG cells into the yolk of a wild-type zebrafish at 48 hpf, while Yang et al. used the same cell line at a volume of 100 cells per embryo at the same stage without a tracking dye [97], [101]. Thus, it is critical to understand the differences that can appear due to the methodological differences and develop a unifying approach for xenografting.

The wet lab part of the research involved in this thesis showed that both the cell amount injected and injection location affect the signal density, area size after injection, and CTCF. With the increased number of cells injected, regardless of the location, it has been shown that higher signal measurements were collected. A prominent and visible gall bladder signal measurement was observed throughout all the images after they were analyzed. Thus, I further investigated the subject and analyzed the gall bladder region only. Still, I couldn't find a significant difference between the groups regarding signal density after looking at their Z-scores. The only groups that showed a significant result were the blastula 300 cells and the yolk 200 cells group. Yet, since

the data has a high standard deviation, an experiment should definitely be repeated to have a clearer idea.

Tracking time of the zebrafish after the xenograft can vary depending on the purpose of the analysis. While there isn't any specific rule regarding the tracking time of the zebrafish after the xenograft, the ZenofishDb Glioma app shows that the most commonly used tumor assessment time is 72 hpi (Table 3.2). Again, using the app, it's been observed that the most preferred tumor injection time is 48 hpf (Table 3.2). So, I based my initial experimental setup for the yolk injections in the same way after the literature search. To be able to compare and have a sensical read on the blastula injections, I conducted a parallel setup and waited till 5 dpf to end all experiments.

The most commonly used injection cell numbers for zebrafish glioma xenografts are shown to be 50-200 cells/embryo in 23 studies and 100-200 cells/embryo in 10 studies based on the extracted information from ZenofishDb Glioma app (Table 3.2). That's why I initially started with the 50-300 range while keeping these studies using different glioma cell lines in mind. After the analysis, although there was a significant signal difference between the lowest cell number group (Blastula 50) and other groups. There wasn't any difference between the yolk 200 cells group and the blastula 100 and 300 cells group. This can result from the cell line used MGG119-GFP or tracking conditions after the injection. Yang et al. showed that after injecting 20, 50, 100, and 200 U87-RFP cells into the yolk of 2 dpf larva, they observed higher cell number-dependent induction of angiogenesis as the injected cell number increased [102]. They performed their analysis after 2 dpi at 4 dpf and found a significant difference between the 20 cells/embryo group and other groups, while there wasn't any other significant difference between any groups [102]. Another study showed that migration and proliferation of Ewing sarcoma can differ between

blastula and larval xenograft models [103]. After injecting TC32 EWS cells into the yolk of 2 dpf zebrafish larva and blastula, they observed that migration of cells from yolk was first observed at 4 dpi in the yolk injection group, whereas it wasn't till 8 dpi that the movement was observed for the blastula group [103]. Also, cell proliferation increased till 3 dpi and plateaued for the 2 dpf group, while for the blastula group, an increase was observed until 5 dpi, and then it started to drop the initial cell volume [103]. These results can suggest that tracking period of the larvae can lead to different interpretations of the results.

The gall bladder function of the zebrafish and humans show similarities as the biliary system in zebrafish comprises both intrahepatic and extrahepatic bile ducts similar to humans. Bile from hepatocytes is transferred to the larger extrahepatic ducts by intrahepatic canals, ultimately directing it toward the gallbladder and intestine [104]. In the hepatocytes, bile acids are produced from cholesterol, stored in the gallbladder, and then transported to the intestine when needed [105]. Fluorescent lipophilic dyes, such as DiI, are commonly employed for labeling neurons through retrograde and anterograde methods, effectively staining cellular membranes [106]. Since they are lipophilic, they can also target biles stored in the gall bladder and cause signal detection.

Another factor that can affect zebrafish health, development, and thus signal during the experiment is the sedative used. Tricaine, or MS-222, is a commonly used anesthetic in zebrafish research. It is mainly used to create an immobile environment so that fish can stay unresponsive during the experiment [107]. This also helps to minimize the stress and potential complications that can occur. On the other hand, long-term exposure to Tricaine can have negative effects on the zebrafish, such as stress and reduced life expectancy [108]. Although there has not been any research suggesting Tricaine shouldn't be used as a sedative during xenograft studies since, if

used excessively, it can reduce the heart rate of an adult zebrafish; it is suggested that it can be coupled with isoflurane [109]. Thus, Tricaine's effect can be ignored in the xenograft I performed as they were neither adult fish nor exposed to Tricaine for a long time.

4.2 Future Perspectives

1. ZenofishDb Glioma application will be updated regularly in the future with the upcoming new research papers by incorporating them into the curation tables as well as using them for text mining.

2. The current version of the ZenofishDb Glioma only deposits the glioma-based xenograft studies. In the future, an adaptation of the code for other cancer types can help researchers from different cancer backgrounds utilize our application for their research. So, the glioma-based curation code will be adjusted for other cancer types to create a more comprehensive zebrafish xenograft archive.

3. The current version of the ZenofishDb Glioma uses a full-text summary of the paper without prioritization of the content in the NLP tab of the app. More specific approaches should be implemented for the extraction of zebrafish-related parts. A more sophisticated filtering is required to pinpoint the zebrafish xenograft portions of the papers. Thus, further development on keyword-based information extraction is needed.

4. In-between-tabs transition can also be implemented in future versions to create a more automated app. This way, after selecting a certain paper from the Data Table tab, immediate pop-ups about its word cloud, NLP data, and abstract can be retrieved automatically.

5. For future updates of the XenofishDb Glioma app, a tab for the protocols and analyses used in the zebrafish xenograft research can be added to provide users with a better understanding of zebrafish research and standardized protocols.

6. ZenofishDb Glioma app will be updated regularly upon checking new literature. Review paper by Pliakopanau and other similar curation platforms will be searched, and missing glioma xenograft papers will be incorporated into future versions.

7. *In vivo,* part of the experiment revealed that yolk injection with 200 cells and blastula injection with 300 cells showed similar results in terms of signal density. Although there was no significant difference between the two findings, further wet lab research can help differentiate the importance of the location and number of cells injected.

8. Other than signal measurement, localization of a gall bladder signal was observed regardless of the injection volume and injection site. Although the results didn't show any significant differences in terms of the signal density between groups, this experiment should be repeated to see if this was a one-time event or an actual biological outcome due to injections.

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