CRISPR Gene Editing Technology Projects at the CRISPR@CEDOC Service

CRISPR/Cas9 gene editing is biotechnological breakthrough that repurposes a bacterial defense system known as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). At CEDOC, we have recently started a CRISPR-based gene editing service (http://cedoc.unl.pt/services-crispr/), and we need to excel at this technique. Thus, in parallel with the projects we develop for clients using the state of the art, we also work CRISPR editing innovations. Below we describe two ongoing projects (the description is vague for copyright purposes).

The Cas9 endonuclease is guided by an RNA to a precise region in the genome. For precise edits, a template DNA is needed from which the genetic information will be copied into the genome. An ingenious improvement was the creation of a Cas9 fusion protein that can be linked to the DNA template, thus increasing its local concentration and near the region to be edited. We are current working on two strategies to further improve this new tool. In these projects, the student will master basic molecular and cellular biology techniques. Each project should be accomplished in 6-8 months.

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Zebrafish larvae genotyping optimization

Key-words: zebrafish; qPCR; PCR; DNA extraction; molecular biology

Zebrafish is an animal research model used in different research lines from cancer to behavior. The number of transgenic and mutant zebrafish lines has accompanied the growth in the number of publications that use this animal as a model. With the latest scientific advances and the democratization of CRISPR technology, the number of zebrafish lines available has grown even more.

One of the daily challenges of a fish facility is to reduce the production of animals to a minimum.

To achieve this goal, it is necessary to optimize new strategies for genotyping a large group of animals, before they have reached adulthood and the reproductive phase.

The objective of this project is to use PCR and qPCR techniques to optimize a protocol of genotyping by environmental PCR based on zebrafish embryonic growing medium.

Our approach will combine the heat-shock DNA extraction method to small samples of embryonic medium, collected from well plates were the embryos have been raised during 48h.

After the PCR protocol optimization process, the aim is to optimize the scale-up protocol using 96 embryos each time, to fulfill the fish facilities daily basis needs.

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The impact of temperature in Zebrafish left-right development

Key-words: Zebrafish; embryonic development; Organ position; Temperature; automatization; screening; microscopy; confocal imaging

The correct position of organs inside the human body (organ situs) is a well-orchestrated process that occurs during embryonic development. The body internal asymmetry starts in a transient organ the "Node" or "left-right organizer" (LRO) during the early stages of embryonic development.

Zebrafish embryos are a very important research animal model regarding embryonic development, especially due to external fertilization and transparency in the early stages of development. This model is widely used in the left-right research field.

Despite all the research already performed in the left-right field, some basic aspects like the impact of temperature variation are not very well understood.

The first task of this project is to evaluate the impact of temperature variation in the left-right organ position, using the zebrafish hearth and liver positions as readouts. For this purpose, we will use embryos that are reporters for sox17 and cmlc2, which will label the liver and pancreas and the heart, respectively.

The proposal is to raise zebrafish embryos in different temperatures during a specific time-window, known to be crucial to the correct organ position, and then evaluate the organ situs at 30hpf using an automated screening microscope that will allow screening 96 embryos in one go.

The second task depends on the first one. If LR defects are indeed found, then we propose to investigate the impact of temperature in membraneless organelles that are present at the base of motile cilia (https://doi.org/10.7554/eLife.38497). By using immunofluorescence and confocal imaging these organelles can be visualized after optimization. Membraneless organelles were proposed to have a relevant role in dynein assembly of the motile cilia and are therefore crucial organelles for motile cilia function. We hypothesize that temperature might affect these organelles even before cilia are formed and for that reason, cilia may not move properly at the time-window they must move to create a fluid flow towards the left side of the embryo, thereby left-right might be severely affected at temperatures different from 28 degrees Celsius.

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In vivo electroporation as a delivery method for generation of transgenic and mutant zebrafish

Key-words: zebrafish; gene-editing; electroporation; molecular biology;

Transgenic and mutant zebrafish strains are important models for biomedical research, including neurosciences, immunology, developmental biology, oncology. There is a wide range of zebrafish transgenesis and gene editing technologies. However, methods to deliver the exogenous components of these systems (DNA, RNA, proteins, synthetic oligos) continue to be limited.

Microinjection into one-cell stage embryos is the most widely used method, but it has a low success rate for the integration of large constructs into the host's genome, and the integration is often associated to mosaicism. To address these constraints, we are testing electroporation in gametes and in one-cell stage embryos as alternative delivery methods for the generation of transgenics and mutants. In vivo electroporation has been successfully used for this purpose in other animal models. Yet, the only protocols available for zebrafish, so far, are for the delivery of macromolecules into targeted regions of larvae or adults, for temporally and spatially controlled study of gene function.

The main goals of this project are to: (1) develop protocols for electroporation of zebrafish gametes and one-cell stage embryos, using NEPA-21 custom-designed electroporation chambers; (2) use the optimized protocols to generate transgenic and/or mutant zebrafish, by delivering plasmid DNA, BACs and/or Crispr-Cas9 components; (3) assess the efficiency of each protocol in terms of construct integration rate and germline transmission; and (4) compare the efficiency of electroporation versus microinjection in gametes and fertilized eggs.

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