

# Perspective on automated *in vivo* drug screening using the *chodl* mutant zebrafish line

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Since the early 2000s, the popularity of zebrafish in *in vivo* drug screening has shown a substantial increase. The zebrafish has become an important screening tool covering a wide range of tissue-specific pathologies and diseases/disorders. Nowadays phenotypic-based screening is favoured over target-based screening approaches, because it can identify active drugs/small molecules in the absence of a known target or suspected mechanism of action. Phenotypic screens can identify not only compounds with a positive effect, but also compounds with off-target effect. Another advantage of whole-organisms screening is the bioavailability of compounds, and the fact that *in vivo* experiments will allow the normal metabolism of these compounds by the living organisms. Overall, this type of approach has boosted the discovery of higher quality compounds compared to *in vitro* target-based screens (Zhang and Peterson, 2019).

## Advantages of zebrafish for drug screening:

All phenotypic screens carried out in cell culture are limited to cell-autonomous phenotypes and to end points that can be easily observed in culture systems. Hence, the necessity becomes evident to have a model that shows complex biological processes and a system of internal organs that potentially metabolises compounds- an intact, living organism. Such an organism is the zebrafish (*Danio rerio*), a small tropical fish that is native to Asia. Zebrafish share a high gene homology with humans, and many of the genes associated with various human diseases have a zebrafish counterpart. Because of this high genetic homology and human-like tissues and organs, zebrafish can be used to study the mechanisms of various human diseases. Zebrafish are very easy to handle, and the cost of maintenance is low compared to other medium-size models, like rodents. The embryos and larvae are optically transparent making it easy to follow internal organ development after any genetic manipulation (CRISP/Cas9, morpholino injections) or pharmacological treatment by non-invasive imaging techniques. Nowadays the availability of various transgenic reporter lines makes the analysis of cell/structure of interest very easy. Zebrafish produce a large number of offspring (200–300), which in

turn can be translated into a high number of compounds/small molecules being tested in parallel. Thus, large clutch size and ease of drug administration (direct administration in water) make zebrafish an excellent tool for automated/semi-automated medium- to high-throughput drug screening.

## The chondrolectin mutant line, a model for motor neuron diseases:

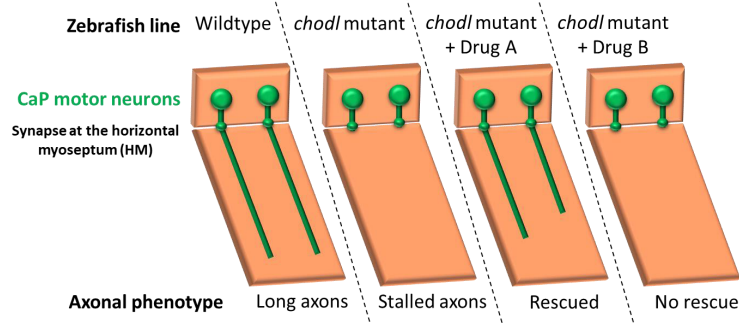
The generic term “motor neuron diseases” refers to a clinically heterogeneous group of developmental and neurodegenerative disorders, characterized by the progressive loss of upper motor neurons, lower motor neurons or both, associated with muscular atrophy or spasticity. Numerous zebrafish lines are now available to study various aspects of the neurodegenerative disorders (mutants, gain- and loss-of-function models, overexpression models) (Wang et al., 2021). One of our main lines of research for our group is compound screen in zebrafish model of motor neuron disease, like spinal muscular atrophy (SMA). SMA is characterized by degeneration of lower motor neurons due to a deletion in survival motor neuron 1 gene (*SMN1*), which leads to downstream dysregulation of various genes (142 genes). One of the earliest dysregulated genes is chondrolectin (*chodl*) (Zhang et al., 2008; Bäumer et al., 2009), a type-I transmembrane C-type lectin protein expressed by zebrafish motor neurons (Zhong et al., 2012) and fast motor neurons in mice (Enjin et al., 2010). Interestingly, overexpression of *chodl* in a zebrafish SMA depletion model is able to partially rescue the motor neuron axonal patterning defects (truncated axons, excessive branching, missing axons) caused by SMN depletion (Sleigh et al., 2014). By generating a *chodl* mutant line we showed in an earlier study that chondrolectin is important in neuromuscular synapse differentiation in zebrafish and mice, and in synapse-dependent motor axon growth in zebrafish (Oprea et al., 2019). The *chodl* mutant line displays stalled CaP (caudal primary) motor axons at the horizontal myoseptum and various synaptic defects (Oprea et al., 2019), suggesting a role of *chodl* in synapse maturation and stabilization (Figure 1). Thus, synapse destabilization observed in the early phase of SMA might correlate with chondrolectin dysregulation at same stage.

Due to the role of chondrolectin in neuromuscular synapse stabilization, we aimed at identifying synapse-stabilizing compounds using the chondrolectin mutant line, which we paired with the powerful VAST (Vertebrate Automated Screening Technology) Bioluminescence screening platform (Oprea et al., 2021). The robust phenotype of the *chodl* mutant line (stalled motor neurons at the horizontal myoseptum) makes it a valuable resource for identifying compounds relevant not only for SMA but also for other motor neuron diseases.

## VAST Bioluminescence screening platform, a boost for drug discovery for neurodegenerative disorders:

The necessity of screening more libraries/compounds in a short period of time has grown over the years. In this respect, the VAST technology has proven to be a big asset, because this automated/semi-automated system can load/unload the zebrafish embryos or larvae and image them. The speed and accuracy of the VAST Bioluminescence platform outstands the “classical work”, where 8 to 10 hours would be spent to manually perform all the necessary tasks (mounting, imaging) only to screen 20 compounds, compared to just 4 hours using the VAST screening platform. In the past, the VAST Bioluminescence platform has been used to screen older embryos and larvae [48 hours post-fertilization (hpf), 72 hpf and 96 hpf] (Early et al., 2018; Ansar et al., 2019). By refining several parameters for loading/unloading, detection and imaging on the VAST Bioluminescence, we were able to screen younger (28–30 hpf) and less pigmented zebrafish embryos than previously possible. Thus, biological processes taking place in earlier developmental stages can now be assessed much quicker. Nowadays, commercially available libraries containing hundreds of small molecules (biologically active compounds with low molecular weight), can be tested in a short period of time (months). For example, in a couple of months, we have tested over 900 compounds on our *chodl* mutant (around 50 screening days/1 person).

**The road to the ideal quantification:** To narrow down a list of compounds (“hits”), likely influencing your process of interest, can be a difficult task, making it challenging to find the perfect way to analyse the results. It takes time to identify the best parameters to be measured and quantified. In our case, the phenotype of the *chodl* mutant line has the advantage that more than 75% of the CaP motor axons are stalled at the horizontal myoseptum (Figure 1). Thus, we developed a two-stage approach to quantify the results. In the first step, we scored the number of CaP motor neurons that passed



**Figure 1 | Schematic representation of the primary CaP motor neurons in zebrafish.**

The axonal phenotype in wild-type (axons grow beyond the horizontal myoseptum) and in *chodl* mutant (axons are stalled at the horizontal myoseptum) are shown. A drug screening can identify compounds that can fully or partially restore the axon growth in the *chodl* mutant line (rescued phenotype).

the horizontal myoseptum, and in the second step we quantified the total axon length of the CaP motor axons. This second parameter is necessary because although the *chodl* phenotype can be rescued by various compound/small molecules, the total length of CaP motor axons can be variable such that more robust effects can be detected by axon length quantification, rather than scoring. The length of CaP motor axons is relevant for the entire musculature innervation. Out of 982 compounds screened, 12 induced an increase in the number of motor axons that grow beyond the horizontal myoseptum, and out of these 12 only 4 compounds induced a statistically significant increase in the total axon length. Our hit rates are similar to other screens in zebrafish (1.22% and 0.40%). This clearly indicates that our approach is valid and by having a 2-step quantification approach we can reduce the list of potential candidates. In this way, we also improved the quality/relevance of the candidates carried further. Because our aim was to find compounds that stabilized synapses, we introduced a 3rd parameter to be analyzed, which was analyses of synapse integrity/maturity. We showed that only 2 compounds were able to rescue some of the synaptic defects of the *chodl* mutant.

**Introducing a 2<sup>nd</sup> model in the screening paradigm:** Many chemical screens usually rely on one single model to identify relevant compounds for a certain disease/disorder, an approach that can be limited in biological relevance. There is the need to test the compounds on several similar or related models (mutant lines) to fully understand its effectiveness. A secondary related model can offer a different perspective and shed some light in the possible mechanisms of action of the compounds. Because *chodl* is a key factor downstream of SMN pathway and is linked to spinal muscular atrophy, the UBA1 model of SMA was chosen as a secondary model in our screening paradigm. The UBA1 model is a chemical-induced model, in which

UBE1-41 inhibitor blocks the activity of UBA1 enzyme (ubiquitin-like modifier-activating enzyme 1). Compared to the *chodl* mutant the UBA1 model displays an array of axonal abnormalities from truncated or missing axons to severe branching. We have shown that the top candidate from the screen on *chodl* mutant fish, dipyrindamole, was able to partially rescue the axonal defects in the UBA1 model. Hence, accumulating evidence suggest that dipyrindamole has neuroprotective properties, which could include synapse protection, as well.

In conclusion, the VAST BioImager can be used for a variety of zebrafish models from neural to non-neural *in vivo* models, providing those cells of interest are transgenically labeled. Furthermore, choosing a two-model system screen over one-model can be beneficial and strengthen the robustness of the results.

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