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Development of a high-throughput tailored imaging method in zebrafish to understand and treat neuromuscular diseases

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The zebrafish (*Danio rerio*) is a vertebrate species offering multitude of advantages for the study of conserved biological systems in human and has considerably enriched our knowledge in developmental biology and physiology. Being equally important in medical research, the zebrafish has become a critical tool in the fields of diagnosis, gene discovery, disease modeling, and pharmacology-based therapy. Studies on the zebrafish neuromuscular system allowed for deciphering key molecular pathways in this tissue, and established it as a model of choice to study numerous motor neurons, neuromuscular junctions, and muscle diseases. Starting with the similarities of the zebrafish neuromuscular system with the human system, we review disease models associated with the neuromuscular system to focus on current methodologies employed to study them and outline their caveats. In particular, we put in perspective the necessity to develop standardized and high-resolution methodologies that are necessary to deepen our understanding of not only fundamental signaling pathways in a healthy tissue but also the changes leading to disease phenotype outbreaks, and offer templates for high-content screening strategies. While the development of high-throughput methodologies is underway for motility assays, there is no automated approach to quantify the key molecular cues of the neuromuscular junction. Here, we provide a novel high-throughput imaging methodology in the zebrafish that is standardized, highly resolutive, quantitative, and fit for drug screening. By providing a proof of concept for its robustness in identifying novel molecular players and therapeutic drugs in giant axonal neuropathy (GAN) disease, we foresee that this new tool could be useful for both fundamental and biomedical research.

KEYWORDS

zebrafish model, imaging methodology, neuromuscular system, disease mechanisms, drug screening

Introduction

Over the last 30 years, the zebrafish (*Danio rerio*) has become the model of choice for many fundamental and translational research studies. With about 70% ortholog genes shared with the human genome (Barbazuk, 2000; Howe et al., 2013), the zebrafish is largely used in the fields of embryology, pharmacology, and toxicology to expand our knowledge on complex biological processes and accelerate the emergence of precision medicine (MacRae and Peterson, 2015). It has also become a preferred species for the modeling of various diseases, including cancer, and cardiovascular, metabolic, and neuromuscular diseases (NMDs) (Seth et al., 2013; Patton et al., 2021).

The success of this animal model largely relies on the numerous advantages it offers to the research community. Regarding practical aspects, the husbandry of zebrafish is relatively simple and inexpensive. Its high fertility rate (∼200 embryos per clutch), rapid and external development, and transparent skin allow direct access to all developmental stages from embryo (one-cell zygote at 0 h post-fertilization, hpf, to hatching at 2–3 days post-fertilization, dpf), to larva (from 3 to 29 dpf) and juvenile (until 6 weeks). As a consequence, zebrafish presents many advantages: (i) genetic manipulation can be achieved at an early stage of embryonic development (injection of antisense oligonucleotides/mRNA for transient effects, or CRISPR agents for the creation of stable transgenic lines), (ii) transparency of embryos allows for the visualization of internal structures and networks at the level of the whole organism, (iii) small-size (0.9–3.5 mm) zebrafish embryos can be kept in 96- or 384-well plates, and (iv) chemical compounds can be administered by simply adding to water (Zon and Peterson, 2005). In practice, the study of zebrafish is cost-effective and compatible with large-scale/high-throughput pharmacological screening (Rennekamp and Peterson, 2015), explaining why it has become the leading model organism for chemical screening and drug discovery.

The zebrafish model has particularly proven to be valuable in vertebrate neuromuscular system studies, which have deepened our knowledge in normal axonal/muscle synapse development and degeneration (Guyon et al., 2007; McLean and Fetcho, 2008; Li et al., 2017) and in identifying the deficits underlying neuromuscular pathologies (Panzer et al., 2005, 2006). The locomotion-sustaining human neuronal circuitry, which is highly conserved in zebrafish, was characterized, thanks to this species (Kimmel et al., 1995; Beattie, 2000). In particular, studies using this model contributed to the identification of molecular signals required for the establishment of motor networks (Berg et al., 2018), neuromuscular junction (NMJ) development, NMJ maintenance, synaptogenesis (Jing et al., 2009, 2010; Banerjee et al., 2011), and identification of muscle precursor types (Devoto et al., 1996).

With the ease of genetic manipulation, various transgenic zebrafish lines represent powerful models to study human diseases, in an era where whole-genome/-exon sequencing generates big data of genetic variants in patients (Pipis et al., 2019; Westra et al., 2019; Herman et al., 2021). In this regard, the zebrafish can be extremely valuable in (1) discovering the disease gene among potential candidates, (2) identifying pathological variants of a known gene, and (3) deciphering pathological mechanisms (McCammon and Sive, 2015; Vaz et al., 2019).

Here, we first describe the key events in zebrafish neuromuscular system development and its resemblance to human counterpart. We then briefly present how the creation of disease models of the neuromuscular system contributes to the understanding of this specific synapse in health, as well as the underlying mechanisms in disease. We then focus on the current behavioral and imaging-based techniques used in the zebrafish, putting in perspective the need to generate tools with greater precision to enable a deeper exploration of the neuromuscular system and to apprehend specificity in disease, an essential aspect for personalized medicine. Finally, we present a novel quantitative and standardized imaging methodology with a proof of concept for its effectiveness in identifying both novel regulators of the neuromuscular system and therapeutic drugs using high-throughput screening.

Zebrafish is a model of choice to study the motor system in human

At the cellular level, the NMJ is made up of the same components across species (**Figure 1A**). It is formed by two entities: the presynaptic motor neuron (MN) of the nervous system and the postsynaptic muscle fiber, which are separated by a synaptic cleft. The transmission of electrical input along the MN axon is achieved by a spread of action potentials. Upon activation, the fusion of vesicles in MN terminals results in a release of acetylcholine (ACh) into the synaptic cleft, which activates postsynaptic nicotinic ACh receptors (AChRs) in the postsynaptic muscle fibers (Kummer et al., 2006). This event depolarizes the muscle cell and triggers calcium release from the sarcoplasmic reticulum to initiate muscle contraction. Many molecular, histological, and ultrastructural features of NMJ development and integrity are well conserved between mammals and zebrafish (Swenarchuk, 2019). Among other examples, the key role of AChR pre-patterning in guiding MN terminals to the muscle fibers was discovered in the zebrafish model (Panzer et al., 2005), as well as the role of the Wnt pathway in this process (Jing et al., 2010).

The skeletal muscle, which is the only muscle tissue voluntary controlled by the central nervous system, is closely related in zebrafish and humans, at histological, biochemical, and ultrastructural levels (Luna et al., 2015). These similarities include the preservation of two sets of MNs, two main types of

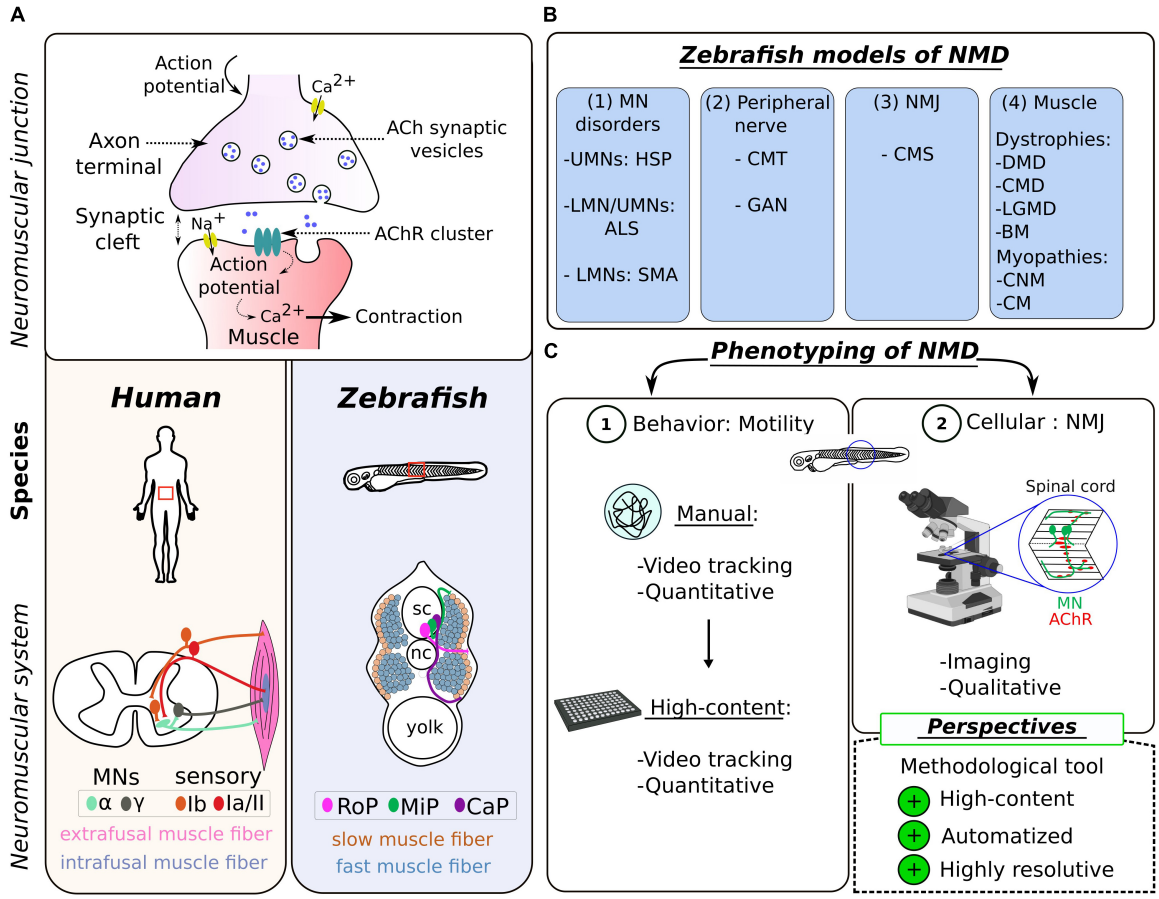

FIGURE 1
Zebrafish as a model of choice to study the neuromuscular system in human. **(A)** Schematic representation of the neuromuscular synapse (top) and the topographical differences (bottom) between the zebrafish and the human species. **(B)** Table summarizing the different NMD pathologies modeled in zebrafish, categorized into four groups according to the first disease target (see **Table 1** for details). UMN, upper motor neuron; LMN, lower motor neuron; HSP, hereditary spastic paraplegia; ALS, amyotrophic lateral sclerosis; SMA, spinal muscular atrophy; CMT, Charcot–Marie–Tooth; GAN, giant axonal neuropathy; NMJ, neuromuscular junction; CMS, congenital myasthenic syndrome; DMD, Duchenne muscular dystrophy; CMD, congenital muscular dystrophy; LGMD, limb–girdle muscular dystrophy; BM, Bethlem myopathy; CNM, centronuclear myopathies; CM, congenital myopathy. **(C)** Scheme summarizing the common tools for NMD phenotyping in the zebrafish, divided into two levels of investigation: (1) the locomotion at the behavioral level and (2) the visualization of the NMJ at the cellular level; and highlighting the methodological challenge presented as perspectives in this review.

muscle fibers (slow and fast), the dystrophin-associated protein (DAPC) complex, and the excitation–contraction coupling system (Berger and Currie, 2012). Differences are restricted to morphological variables such as MN axon diameters and average area of AChR clusters (Jones et al., 2017; Boehm et al., 2020), and the specificity/subclassification of muscle fibers, which have their source in an adaptation of locomotor behavior. In humans, there are two major types of spinal MNs located in the anterior horn of the spinal cord, differentiated according to the size of the soma and their innervation (**Figure 1A**): α-MNs and γ-MNs (Kanning et al., 2010; Manuel and Zytnicki, 2011). The α-MNs are the most abundant type of MNs in mammals. They innervate extrafusal muscle fibers classified into different subtypes, whose contractile properties differ: slow-twitch, fatigue-resistant (S), and fast-twitch fibers, which are further subdivided into fatigue-resistant (FR) and fatigable (FF) fibers (Brooke and Kaiser, 1970; Burke et al., 1971). The γ-MNs innervate intrafusal muscle fibers responsible for muscle tone and proprioception. Zebrafish spinal MNs are also divided into two groups, differentiated in two distinct waves during development: primary motor neurons (pMNs) and secondary motor neurons (sMNs) innervating specific musculature (Myers, 1985; Eisen et al., 1986). There is also a subclassification of primary motor neurons (pMNs) in each hemisegment, originally designated according to their specific location as caudal primary motor neuron (CaP); middle primary motor neuron (MiP), and rostral primary motor neuron (RoP) (**Figure 1A**), innervating different muscle fiber territories. Motor neurons from the second wave (sMNs) are considered to be equivalent to α-MNs in humans (Beattie et al., 1997).

They have small-size somas, are more numerous, and innervate both deeper fast-twitch muscle and superficial slow-twitch muscle fibers (Menelaou and McLean, 2012; Bello-Rojas et al., 2019). It is worth noting that the proportion of fast muscles is much higher in zebrafish than in mammals, an adaptative response to the need to produce bursting and rapid powerful movements.

Zebrafish mimics pathologies of the neuromuscular system

The genome is highly conserved between the human and zebrafish species, with a homology for over 80% of disease-causing genes (Howe et al., 2013). This evolutionary characteristic fostered the generation of zebrafish mutants to model pathologies of the human neuromuscular system (Pappalardo et al., 2013). Strategies of transient genetic manipulations using microinjection (Eisen and Smith, 2008) (gene inactivation to model recessive diseases or mRNA overexpression for dominant pathologies), followed by genome editing (Gaj et al., 2013; Schmid and Haass, 2013), have been successfully used to assess the significance of pathological mutations associated with neurodevelopmental and neurodegenerative conditions, including NMDs.

Here, NMDs are defined as pathologies inducing muscle weakness and atrophy (Efthymiou et al., 2016). Thus, NMDs cover a large group of diseases of heterogeneous etiology, affecting the muscles and/or the nerves and comprising (1) motor neuron disorders [e.g., amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), and hereditary spastic paraplegia (HSP)], (2) disorders with a component in the peripheral nerves [e.g., Charcot–Marie–Tooth disease (CMT) and giant axonal neuropathy (GAN)], (3) disorders directly affecting the NMJ [e.g., congenital myasthenic syndrome, (CMS) and Lambert–Eaton syndrome], and (4) primary disorders of the muscle (myopathies and muscular dystrophies) (**Figure 1B**).

While we present numerous zebrafish models generated to model NMDs (**Table 1**), we do not intend to describe them all. Instead, we focus on highlighting some features as evidence for great relevance and advantage of the zebrafish research model in these types of studies:

- Zebrafish models of the same disease group, for example, of the MNs (ALS, SMA, and HSP) induce similar deficits, including loss of MNs, aberrant axonal outgrowth, loss of neuromuscular connectivity, muscle denervation, and defective motor performance. The study of these parameters, as easily performed in the zebrafish, enriches our knowledge of the molecular players in the settings and maintenance of the neuromuscular system.
- In a context of diseases with multiple genetic origins (most of the clinical groups described in **Table 1**), the zebrafish model provides the versatility to validate gene discovery and pathogenic variants using fast and penetrant functional characterization (see Patten et al., 2014; Naef et al., 2019 for review).
- For several diseases, such as GAN (Arribat et al., 2019) and HSP (als2, Gros-Louis et al., 2008), zebrafish models are the first to reproduce the severity of motor and neuromuscular symptoms, where mouse models have so far failed (Cai et al., 2008; Dequen et al., 2008; Ganay et al., 2011). This reinforces the notion of the conservation of the neuromuscular system between the zebrafish and human species.
- The zebrafish represents a highly relevant model for therapeutic development as its physiology is well conserved with human. For example, the zebrafish is an excellent model for muscle pathologies (Jagla et al., 2017), more adapted than mouse (Berger and Currie, 2012). Models with mutations in the zebrafish dystrophin gene (*dmd* and *sapje* models) of DMD have been developed (Bassett and Currie, 2004; Guyon et al., 2009), whose robustness of phenotypes (muscle lesions and birefringence, almost 100% penetrant) has led to the development of a therapeutic strategy for human patients (Johnson et al., 2013; Farr et al., 2020), which is now in phase 3 in clinical trial.

Current methods to study the neuromuscular system in health and in neuromuscular diseases

The phenotyping of zebrafish models of NMDs and, more generally, the study on the regulation(s) of the neuromuscular system rely on two levels of analysis: the locomotion at the behavioral level (Granato et al., 1996) and the visualization of the NMJ at the cellular level (Fetcho, 2007). We present the most common tools used in zebrafish, highlighting their advantages and their limitations (**Figure 1C**).

Locomotion assay: First level of investigation

The zebrafish larvae serve as a powerful model to dissect locomotor patterns as their swimming behavior is defined by sequences of stereotyped movements (Wolman and Granato, 2012). At 5 dpf, when the neuromuscular system is established, the zebrafish is capable of fully autonomous and spontaneous movement. At this stage, motility assays have the advantage to be purely motor, contrary to other locomotor tests performed on younger zebrafish embryos, which include a sensory component (e.g., evoked response to stimuli at 48 hpf). The analysis

TABLE 1 Zebrafish models of NMDs and their distinct behavioral and cellular phenotypes.

	Disease	Gene		Zebrafish model	Locomotor phenotype	Cellular phenotype	References
		Human	ZF				
Motor neuron	**Hereditary spastic paraplegia (HSP):** Upper MN	*SPG3A*	*spg3a*	*spg3a* MO	Impaired touch-evoked escape response (72 hpf)	Abnormal axonal branching and outgrowth	Fassier et al., 2010
		SPG4	*spg4*	*spg4* MO	Impaired locomotion and endurance (5 dpf)	Abnormal axonal outgrowth/ NMJ defects (reduction in SV2 and BTX colocalization)	Wood et al., 2006
		SPG8	*spg8*	*spg8* MO	N/D	Possible defects in axonal outgrowth	Valdmanis et al., 2007
		SPG11 *SPG15*	*spg11* *spg15*	*spg11* MO *spg15* MO	N/D	Abnormal axonal branching	Martin et al., 2012
		SPG39/ PNPLA6	*spg39*	*pnpla6* MO	N/D	MN loss/Abnormal axonal branching and outgrowth	Song et al., 2013
		SPG42/ SLC33A1	*slc33a1*	*slc33a1*MO and mutant mRNA	N/D	Abnormal axonal branching and outgrowth	Lin et al., 2008; Mao et al., 2015
		SPG46/GBA2	*gba2*	*gba2* MO	Impaired touch-evoked escape response (48 hpf)	Possible defects in axonal outgrowth and branching	Martin et al., 2013
		SPG53/ VPS37A	*vsp37a*	*vsp37a* MO	Impaired touch-evoked escape response (96 hpf)	N/D	Zivony-Elboum et al., 2012
		SPG76/ CAPN1	*capn1a*	*capn1a* MO	N/D	N/D	Gan-Or et al., 2016
		SPG80/ UBAP1	*ubap1*	*ubap1*$^{-/-}$ and mutant RNA	N/D	Possible defects in axonal outgrowth	Farazi Fard et al., 2019
		IAHSP/ ALS2	*als2*	*als2* MO	Impaired touch-evoked escape response (48 hpf)	Possible defects in axonal outgrowth	Gros-Louis et al., 2008
	Amyotrophic lateral sclerosis (ALS): Upper and lower MN	*SOD1*	*sod1*	*sod1* mutant mRNA	N/D	Abnormal branching and outgrowth	Lemmens et al., 2007
				sod1 mutant mRNA	Impaired locomotion upon light stimuli (48 hpf)	Abnormal axonal branching and outgrowth	Robinson et al., 2019
				sod1^{G93R}	Decreased endurance and partial paralysis (10 months)	MN loss/NMJ defects (reduction in SV2 and BTX colocalization)/muscle defects (caliber and degeneration)	Ramesh et al., 2010; Benedetti et al., 2016
				sod1^{T70I}	Decreased endurance (20 months)	MN loss/NMJ defects (reduction in SV2 and BTX colocalization)	Da Costa et al., 2014
				sod1^{G93A}	Impaired locomotion and endurance (5 months)	MN loss/Abnormal axonal branching and outgrowth/impaired NMJs	Sakowski et al., 2012
		TDP-43/ TARDBP	*tdp-43*	*tdp-43* MO and mutant mRNA	Impaired touch-evoked escape response (48 hpf)	Abnormal axonal branching and outgrowth	Kabashi et al., 2010
				tdp-43 mutant mRNA	N/D	Abnormal axonal branching and outgrowth	Laird et al., 2010
				tardbp$^{-/-}$	N/D	Abnormal axonal outgrowth/disorganization of myofibrils	Schmid et al., 2013
				tardbp^{fh301}	Impaired escape response (5 dpf)	Abnormal axonal outgrowth	Hewamadduma et al., 2013
		FUS	*fus*	*fus* MO and mutant mRNA	Impaired touch-evoked escape response (48 hpf)	Abnormal axonal branching and outgrowth	Kabashi et al., 2011
				fus MO and mutant mRNA	Impaired touch-evoked escape response (48hpf)	Reduced CaP rheobase current/NMJ defects (reduction in SV2 and BTX colocalization)	Armstrong and Drapeau, 2013
		C9ORF72	*c9orf72*	*c9orf72* MO	Impaired touch-evoked escape response (48 hpf) and spontaneous locomotion (96 hpf)	Abnormal axonal branching and outgrowth	Ciura et al., 2013

(Continued)

TABLE 1 (Continued)

	Disease	Gene		Zebrafish model	Locomotor phenotype	Cellular phenotype	References
		Human	ZF				
				c9orf72 mutant mRNA	N/D	Abnormal axonal branching and outgrowth	Swinnen et al., 2018
				$c9orf72^{89HRE}$	Center avoidance behavior (5 dpf) and decreased endurance (8 months)	MN loss/Muscle atrophy	Shaw et al., 2018
				$c9orf72^{100GR}$	Impaired locomotion (7 dpf)	Abnormal axonal branching and outgrowth/Increase apoptosis in the spinal cord	Swaminathan et al., 2018
				c9orf72 MO	Impaired touch-evoked escape response (48 hpf)	Abnormal axonal branching and outgrowth	Yeh et al., 2018
				$c9orf72^{miR}$	Impaired locomotion (6 dpf, 12 months)	MN loss/Reduced AChR clusters at NMJ/Muscle atrophy and defects (weak mEPCs, thin diameter)	Butti et al., 2021
		SQSTM1	*sqstm1*	*sqstm1* MO	Impaired touch-evoked escape response(48 hpf)	Abnormal axonal branching and outgrowth	Lattante et al., 2015
	Spinal muscular atrophy (SMA): Lower MN	*SMN1,2*	*smn1*	*smn1* MO	N/D	Abnormal axonal outgrowth	McWhorter et al., 2003; Winkler et al., 2005; Gassman et al., 2013; See et al., 2014
				smn^{G264D} smn^{Y262X} smn^{L265X}	N/D	Lack of pre- and post-synaptic protein co-localization/decrease in SV2 protein at the NMJ	Boon et al., 2009
			smn2	$smn^{Y262-/-}$	Impaired locomotion (9 dpf)	Abnormal axonal branching and outgrowth	Hao et al., 2011, 2012
		CHODL	*chodl*	*chodl* MO	N/D	Abnormal axonal branching and outgrowth/Reduced myotome innervation	Zhong et al., 2012
				$chodl^{-/-}$	Impaired touch-evoked escape response (3 dpf)	Prolonged stalling of the CaP axons at the HM/Impaired synaptogenesis	Oprişoreanu et al., 2019, 2021
Peripheral nerve	**Charcot-Marie-Tooth disease (CMT2A)**	*MFN2*	*mfn2*	*mfn2* MO	Impaired touch-evoked escape response (48 hpf)	Abnormal axonal outgrowth/U-shaped somites and decreased muscle width	Vettori et al., 2011
				$mfn2^{L285X/L285X}$	Impaired locomotion and endurance (3 months)	NMJ defects (reduction in pre- and post-synaptic area)	Chapman et al., 2013
		LRSAM1	*lrsam1*	*lrsam1* MO	Impaired touch-evoked escape response (48 hpf)	N/D	Weterman et al., 2012
		DNM2	*dnm2*	*dnm2* MO and mutant mRNA	Impaired touch-evoked escape response (72 hpf)	MN loss/Abnormal axonal branching/NMJ defects (AchR clusters)/increases muscle fiber diameter	Bragato et al., 2016
				$dnm2^{G537C}$	Impaired touch-evoked escape response (48 hpf)	Muscle defects (structural, diameter)	Zhao et al., 2019
	Giant axonal neuropathy (GAN)	*GAN*	*gan*	*gan* MO and $gan^{-/-}$	Impaired touch-evoked escape response (72 hpf) and spontaneous locomotion (5 dpf)	MN loss (specification defect)/Abnormal axonal branching and outgrowth/NMJ defects/Muscle deficits (U-shape somites, structure)	Arribat et al., 2019
Neuromuscular junction	**Congenital myasthenic syndrome (CMS)**	*CHAT*	*chata*	$chata^{tk64}$	Impaired touch-evoked escape response (48 hpf)	No phenotype	Joshi et al., 2018
		DOK7	*dok7*	*dok7* MO	Impaired touch-evoked escape response (48 hpf) and spontaneous locomotion (5 dpf)	Abnormal axonal outgrowth/NMJ defects (abnormal AChR prepatterning, reduction in size)/disorganization of slow muscle fiber	Müller et al., 2010; McMacken et al., 2018
		SLC25A1	*slc25a1*	*slc25a1* MO	Impaired touch-evoked escape response (48 hpf)	Abnormal axonal outgrowth	Chaouch et al., 2014

(Continued)

TABLE 1 (Continued)

	Disease	Gene		Zebrafish model	Locomotor phenotype	Cellular phenotype	References
		Human	ZF				
		GFPT1	*gfpt1*	*gfpt1* MO	Impaired touch-evoked escape response (48 hpf)	Abnormal axonal banching/NMJ defect (delayed development)/Muscle deficits (U-shape somites, structure)	Senderek et al., 2011
		MYO9A	*myo9a*	*myo9a* MO	Impaired touch-evoked escape response (48 hpf)	Possible abnormal axonal branching and outgrowth/impaired AChR clustering	O'Connor et al., 2016, 2018
Muscle	**Duchenne muscular dystrophy (DMD)**	*DMD*	*dmd*	*dmd* MO	N/D	Disorganized sarcomeres	Guyon et al., 2003
				sapje	N/D	Muscle defects (lesions, fiber detachment and retraction, structure, decreased active force)	Bassett et al., 2003; Bassett and Currie, 2004; Widrick et al., 2016
				dmd^{pc2}	N/D	Muscle defects (fiber detachment and retraction, degeneration)	Berger et al., 2011; Giacomotto et al., 2013
				sap^{cl100}	N/D	Muscle degeneration	Guyon et al., 2009
	Congenital muscular dystrophy	*ITGA7*	*itgα7*	*itgα7* MO	N/D	Muscle defect (detachment and retraction of muscle fibers, U-shape somite)	Postel et al., 2008
		LAMA2	*lama2*	*caf* $lama2^{cl501/cl501}$	Impaired touch-evoked escape response (72 hpf)	Muscle defects (fiber detachment and retraction, degeneration)	Hall et al., 2007; Gupta et al., 2012
	Bethlem myopathy	*COL6A1/COL6A3*	*col6a1/col6a3*	*col6a1* MO *col6a3* MO	Impaired touch-evoked escape response (48 hpf)	Muscle defects (U-shaped somites, structure, degeneration)	Telfer et al., 2010; Zulian et al., 2014; Radev et al., 2015
	Limb-girdle muscular dystrophy (LGMD) and **Spinal and bulbar muscular atrophy (SBMA)**	*DNAJB6*	*dnajb6*	*dnajb6* MO and mutant RNA	N/D	Muscle defects (fiber detachment, structure)	Sarparanta et al., 2012; Xu et al., 2022
		DAG1	*dag1*	*dag1* MO	Impaired touch-evoked escape response (48 hpf)	Muscle defects (U-shaped somites, fiber detachment, structure, degeneration)	Parsons et al., 2002; Lin et al., 2011; Goody et al., 2012
				$dag1^{V567D/V567D}$	Impaired spontaneous locomotion (7 dpf)	Muscle defects (structure, degeneration)	Gupta et al., 2011
		POPDC3	*popdc3*	*popdc3* MO	N/D	Muscle defect (fiber detachment)	Vissing et al., 2019
		TCAP	*tcap*	*tcap* MO	Impaired spontaneous locomotion (5 dpf)	Muscle defects (U-shaped somites, fiber detachment, structure)	Zhang et al., 2009; Lv et al., 2022
		SGCD	*sgcd*	*sgcd* MO	N/D	Muscle defects (U-shaped somites, structure, degeneration)	Guyon et al., 2005; Cheng et al., 2006
	LGMD and **Miyoshi myopathy (MM)**	*DYSF*	*dysf*	*dysf* MO	N/D	Muscle defects (less-clear V-shape somite, structure, degeneration)	Kawahara et al., 2011
	Dystroglycano-pathies	*FKRP*	*fkrp*	*fkrp* MO	N/D	Muscle defects (U-shaped somites, fiber detachment, structure, degeneration)	Thornhill et al., 2008; Kawahara et al., 2010; Lin et al., 2011; Bailey et al., 2019
				$fkrp^{\Delta 13/\Delta 13}$	Impaired spontaneous locomotion (5 dpf)	Muscle defect (degeneration)	Serafini et al., 2018

(Continued)

TABLE 1 (Continued)

Disease	Gene		Zebrafish model	Locomotor phenotype	Cellular phenotype	References
	Human	ZF				
	FKTN	*fktn*	*fktn* MO	N/D	Muscle defects (U-shaped somites, degeneration)	Lin et al., 2011
	INPP5K	*inpp5k*	*inpp5k* MO	Impaired touch-evoked escape response (72 hpf)	NMJ defect (reduced arborization)/Muscle defects (structure, degeneration)	Osborn et al., 2017
	ISPD	*ispd*	*ispd* MO	N/D	Muscle defects (retracting fibers, structure, degeneration)	Roscioli et al., 2012
	B3GNT1	*b3gnt*	*b3gnt1* MO	N/D	Muscle defects (fiber detachment, U-shaped somites, structure, degeneration)	Buysse et al., 2013
	GTDC2	*gtdc2*	*gtdc2* MO	N/A	Possible muscle defect (U-shaped somites)	Manzini et al., 2012
Myotubular myopathy	*MTM1*	*mtm1*	*mtm1* MO	Impaired touch-evoked escape response (72 hpf)	Muscle defects (fiber detachment, structure, excitation-contraction coupling abnormalities)	Dowling et al., 2009
Centronuclear myopathies (CNMs)	*MTMR14*	*mtmr14*	*mtmr14* MO	Impaired touch-evoked escape response (48 hpf)	Muscle defect (excitation-contraction coupling abnormalities)	Dowling et al., 2010
	DNM2	*dnm2*	*dnm2* mutant RNA	Impaired touch-evoked escape response (72 hpf)	NMJ defects (defects in AChR clustering)/Muscle defects (structure)	Gibbs et al., 2013
Myofibrillar myopathy	*DESM*	*desm*	*desma* MO *desmb* MO	Impaired spontaneous locomotion (4 dpf)	Muscle defects (fiber detachment, structure, decreased active force)	Li et al., 2013
	FLNC	*flnc*	*sot* Mutant *flnca* MO $flnc^{W2710X}$	N/D	Muscle defects (fiber disintegration, structure, degeneration)	Ruparelia et al., 2012, 2016
	ZASP	*zasp*	*cypher* MO	N/D	Muscle defects (U-shaped somites, structure, degeneration)	van der Meer et al., 2006
Congenital myopathy	*RYR1*	*ryr1b*	ryr^{mi340}	Impaired touch-evoked escape response (48 hpf)	Muscle defects (structure, excitation-contraction coupling abnormalities)	Hirata et al., 2007

Here we focus on neuromuscular phenotypes and excluded other phenotypes (cardiac, central nervous system alterations. . .) described in the articles. We also exclude specific form of HSP (MARS, X-linked formed): see Naef et al. (2019) for complete review. ZF, zebrafish; MO, morpholino; N/D, not determined (or data not provided); HM, horizontal myoseptum; mEPCs, miniature endplate currents; BTX, bungarotoxin.

of locomotor activity of larval zebrafish has emerged as a potent tool for phenotypic assessment in the neurosciences and toxicology fields (Basnet et al., 2019). Some commercial tools are available to the community (e.g., Noldus, Viewpoint, or Loligo® Systems): they provide a means to measure different properties of the swimming activity, including quantification of kinetic parameters such as frequency, duration, speed, and total distance traveled within a given time (Noldus et al., 2001). Few academic laboratories developed locomotion-based tracking systems with deeper analysis of the movement patterns of larvae that are suitable for high-content analysis (Colwill and Creton, 2011; Zhou et al., 2014). They were instrumental in providing a better analysis of the sequencing of swimming bouts (Reddy et al., 2022) and in discriminating the great variability in the escape behaviors (Kohashi and Oda, 2008), by measuring the delay, amplitude, duration, frequency, angle, and number of the tail-bending oscillations (Mirat et al., 2013). Overall, the latter methodologies are extremely attractive, translationally relevant, and easily adaptable to high-throughput pharmacological screening strategies but unfortunately still too rarely used by the general academic community.

While important for the comprehension of the locomotor network (Berg et al., 2018), behavioral tests are not systematically used in disease models, as shown in **Table 1**. Still, when performed, they are valuable to quantify the degree of locomotor deficits: less frequent turning and swimming bouts in the SMA zebrafish model (Hao et al., 2012), shorter traveled distance in the ALS zebrafish model (Armstrong and Drapeau, 2013; Robinson et al., 2019), and severe reduction in both total distance and net velocity (Zhao et al., 2019) in CMT and GAN models (Arribat et al., 2019). Nevertheless, these tools present limitations when it comes to grading the severity of dysfunction between NMD types, or for distinct genes within the same group of diseases. This challenge is mainly due to high variability in the settings applied to the motility tests (type, alternation of light/dark stimulation, duration of the assay, etc.). For example, one can note that the great disparity in the locomotor tests used to describe motor deficits in ALS models, specifically in the *SOD1* gene (see **Table 1**). This

aspect has some importance when choice needs to be made to select strong candidates for preclinical studies or discuss the relative contribution of molecular players. Most importantly, current behavioral tests have limited outcomes in defining the functional origin (neuron, NMJ, or muscle) and the cellular signature of individual gene or NMD.

Cellular defects: 2d level to provide specificity

Due to their transparency, zebrafish embryos are particularly suited for cellular imaging (see Pappalardo et al., 2013; Babin et al., 2014; Patten et al., 2014 for review). For the motor system, classic methods are the labeling of the sub-compartments of the NMJ (Panzer et al., 2005) and providing qualitative measures of the integrity and stability of the NMJ. Some pieces of off-line, free, and open-source image processing and analysis software have been used as quantitative tools for the analysis of the NMJ such as axonal length, AChR clustering, and the coefficient of co-localization of NMJ markers (pre- and postsynaptic). Thus, from confocal images, the authors use these methods to (1) measure axonal length [e.g., NeuronJ plugin in ImageJ software (Boon et al., 2009; Robinson et al., 2019; Campanari et al., 2021; Oprişoreanu et al., 2021) or Lucia software (Lemmens et al., 2007)] or (2) define the score for normal versus abnormal (McWhorter et al., 2003; Boyd et al., 2017; Swinnen et al., 2018). Rare laboratories have developed specific metrics for the quantification of motor axon development, as in Smn-deficient zebrafish embryos (Gassman et al., 2013). Overall, the existing analysis tools permit the extraction of quantifiable data of only a limited number (about six or seven) of axons per zebrafish sample. Thus, one important limitation of the current cellular analysis is the difficulty in extracting measurable data for the whole NMJ, especially due to the overlap between pre- and postsynaptic staining. Indeed, unlike other mammalian species such as the mouse, the fish exhibits a spreaded distribution of the motor nerve terminals and a great extent of postsynaptic folding (Slater, 2017), thus particularly challenging to visualize in detail and quantify.

Perspective in cellular imaging

Currently, most cellular studies in zebrafish are conducted manually, using low-throughput imaging techniques. Still, there is a need for standardized, quantitative, and high-resolution techniques to gain in-depth knowledge of the neuromuscular unit and to define specificity of alterations of NMJ (and therefore mechanisms) across pathologies. Moreover, similar to behavioral assays, developing automated tools, compatible with high-throughput screening assays, would be essential to identify and determine the effectiveness of potential drugs in modulating or restoring the neuromuscular system in health and disease. Recent advancements in imaging-based high-content screening technologies for drug discovery and toxicology have mostly found applications in 3D tissue culture or 3D organoid models (Li et al., 2016). Although the zebrafish has earned its place as an efficient model for high-throughput drug screening, the current tools lack a standard pipeline for imaging small organisms. Therefore, designing a quantifiable and standardized methodology with robust assay metrics to quantify parameters of the neuromuscular unit would be critical for both fundamental biology and clinical research.

Development of a novel quantitative imaging methodology to study and treat the neuromuscular system

To address the challenge of standardization and reproducibility of imaging tools in zebrafish, we developed an automated, high-throughput, imaging pipeline in zebrafish, integrating a quantitative analysis of the key parameters of the neuromuscular system: axonal length, AChR clustering, and NMJ synapse. Here, we mainly describe this novel quantitative methodology and briefly illustrate its potency in delineating the specific phenotype and identifying therapeutic drugs in GAN disease.

Our method relies on automated high-resolution image acquisition using multi-well plates (Opera Phenix^{TM} high-content imaging systems, Perkin Elmer) and involves the development of an image processing sequence using Harmony software (v4.9, Perkin Elmer) to prefilter whole-embryo images and exclude regions with artifacts and high background. This protocol allows to define the regions of interest (ROIs) within the spinal cord and make them automatically detectable and accessible to downstream analysis (**Figure 2**).

- Prerequisite: The first prerequisite concerns the establishment of a model of the neuromuscular system or pathology in the zebrafish, whose phenotype is at least partially described in larval stages. It can include a motor component, with a defect in locomotion with a severity ideally similar to that observed in human patients and/or a cellular defect of varying severity in the neuromuscular system.
- Possible applications: The methodology presented here can have a fundamental and/or therapeutic vocation. Indeed, it allows to scrutinize the neuromuscular phenotypes in

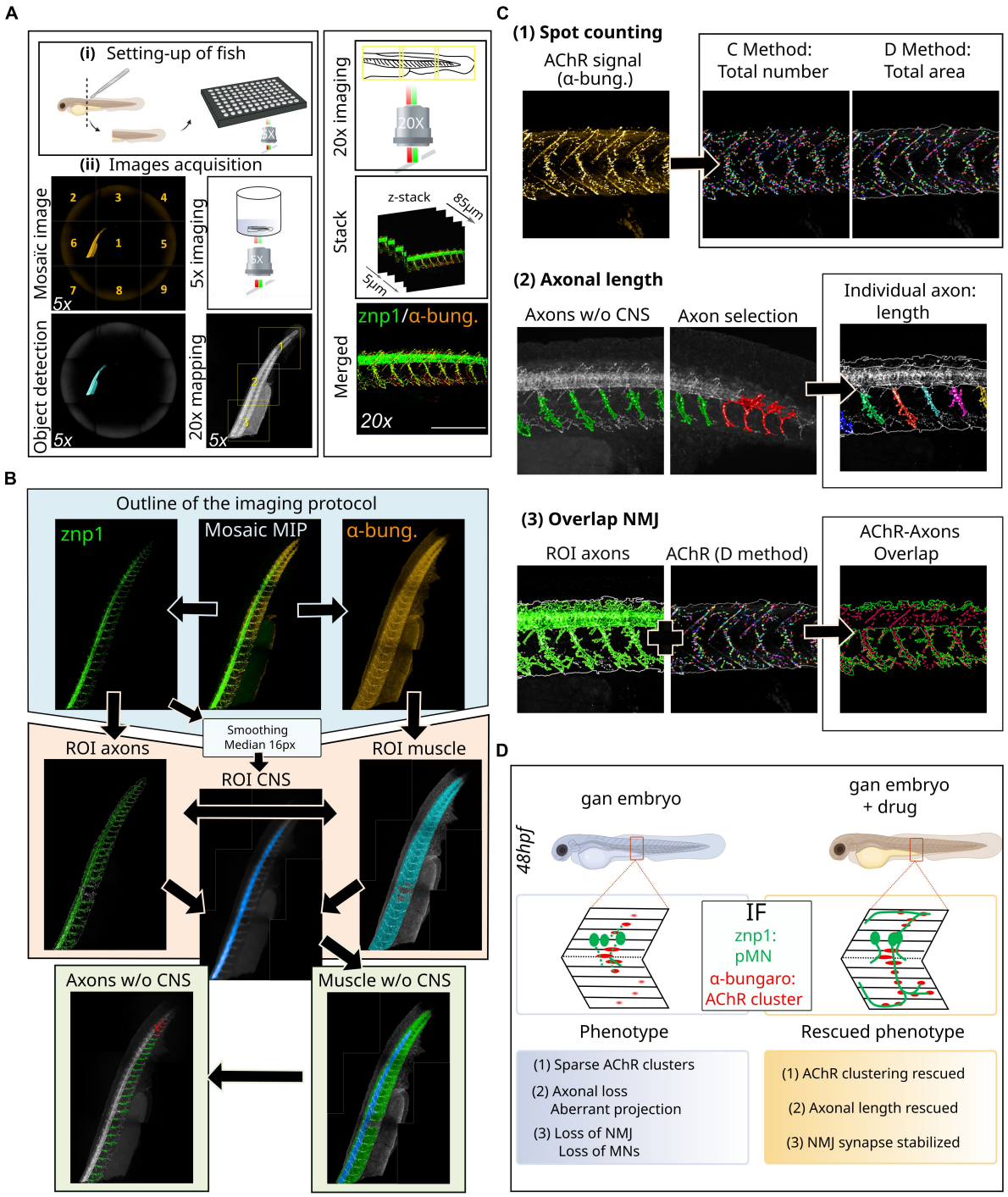

FIGURE 2
Novel methodology for high-throughput imaging of the neuromuscular system in the zebrafish. **(A)** Schematic overview of the setup of 48hpf larvae in 96-well plates (i) for automated detection and image acquisition (ii), from object detection (5x) to stacked images (20x). **(B)** Outline of the imaging and analytic protocol: representative images of NMJ staining (znp1 in green, α-bungarotoxin in red) within the spinal cord of control larvae, from which several steps of ROI segmentation allow to define the different components of the NMJ. **(C)** Representative images of the different filters resulting in the segmentation of AChR clusters from α-bungarotoxin staining and individual axon from the znp1 staining, enabling the quantitative assessment of spot counting (1), axonal length (2), and NMJ overlapping compounds within the spinal cord (3). See the **Supplementary Figure 1** to assess for the quality of the processing tools for each filter: raw data compared to post-analysis pictures at high magnification. **(D)** Validation of this methodology in the zebrafish model of giant axonal neuropathy (*gan*). Detailed coverage of NMJ defects in the gan model and identification of hits restoring three parameters of the neuromuscular system, following a high-throughput screening. α-bung, α-bungarotoxin; pMN, primary motor neuron; MN, motor neuron; NMJ, neuromuscular junction; AChR, acetylcholine receptors; MIP, maximum intensity projections; ROI, region of interest; w/o, without; CNS, central nervous system.

greater detail and to identify therapeutic molecules able to restore them. In the latter, it is recommended to carefully select a chemical library of interest (see Peterson and Fishman, 2011 for review).

- Procedure/Methodology: In the next paragraph, we provide a detailed description of the high-throughput imaging approach, which offers high-resolution images of 3D zebrafish larvae mounted in individual wells in 96-well plates. Then, we detail the development of an image analysis sequence, allowing first to define the different ROIs by using maximum intensity projections (MIP) and then to submit them to different analysis filters to extract measurable neuromuscular parameters. Noteworthy, our methodology on high-resolution images allows the quantification of the NMJ, a challenge in the zebrafish model due to dispersed sites of muscle innervation.

Setting up of fish

Zebrafish embryos of desired aged (see "Advantages of the method" section) are placed in 96-well plates and eventually exposed to small-molecule libraries of compounds that can elicit phenotypic alterations (**Figure 2Ai**). While three larvae represent the most common settings (Rennekamp and Peterson, 2015), we determined that each well can house up to four zebrafish larvae without compromising proper development. On another note, it is recommended to use plates with a thin and flat plastic or glass bottom. Since the interest here is to image a lateral view of the spinal cord, we recommend cutting the embryo at the anterior part of the yolk extension to enable lateral positioning.

Axonal and neuromuscular junction staining

Neuromuscular junctions can be visualized by light microscopy following labeling of both the presynaptic nerve terminal and the postsynaptic acetylcholine receptors (**Figure 2A**). The most commonly used presynaptic antigens, as in other vertebrate models, include anti-neurofilament (e.g., 3A10), anti-synaptotagmin (e.g., znp-1), anti-neurolin (e.g., zn8), and anti-synaptic vesicle glycoprotein 2 (SV2) to fully label both the pre-synaptic axon and nerve terminal. The fluorescently tagged α-bungarotoxin is the most commonly used protein to label acetylcholine receptors in the postsynaptic membranes of the NMJ. Here, we opted to use the znp-1/α-bungarotoxin combination, which ensures specific marking of the axons and NMJ with little background. Primary antibodies are from the following sources: mouse IgG2a anti-synaptotagmin (1:100, znp-1, DSHB) and anti-α-bungarotoxin (1:50, B35451, Invitrogen). We believe the same methodology can be applied to other NMJ markers mentioned earlier. Indeed, the analysis sequences permit the collection of a large number of measurable parameters, as long as the immunostaining is of required quality.

Image acquisition settings of chemical-treated embryos

1. Pre-scan is performed at 5X magnification on the full well surface (nine fields per well with 6% overlap) and red channel only (561 nm) with the widefield mode (**Figure 2Aii**).
2. Use the "*In-the-fly image analysis*" tool to create a mosaic image and "*Find Image Region*" module to detect the whole zebrafish.
3. Use the "*Determine Well Layout*" module to define a 20X re-scan magnification (with 6% overlap) covering the entire object.
4. Re-scan at 20X magnification on whole fish larvae in the confocal mode. Green (488 nm/znp-1) and red (561 nm/α-bungarotoxin) channels are imaged, with a z-stack of 85 μm (5 μm interval).

Definition of regions of interest

5. First, acquire mosaic images (full fish larvae) with MIP for each channel (**Figure 2B**).
6. To automatically identify the two ROI named axons and muscle, we used the "*Find Image Region*" module in green (488 nm/znp-1) and red (561 nm/α-bungarotoxin) channels, respectively. The final ROI muscle was obtained by subtracting seven pixels around the initial region to restrict the analysis to the region of interest and to reduce detection of artifacts.
7. The main challenge is to separate each axon into a different entity. To do this, it is imperative to differentiate the axons from the spinal cord. From the same mosaic images with MIP, we applied smoothing with a median filter of 20 px using the "*Filter Image*" module in the green (488 nm/znp-1) channel. On this new image, we used the "*Find Image Region*" module to find a new ROI: central nervous system (CNS, spinal cord). Then, we used "*Calculate Morphology Properties*" to measure the size and the position of the CNS ROI.
8. Using the "*Find Surrounding Region*" module, the CNS ROI was subtracted from the fish body area

(ROI muscle), to create a new surrounding region: body without CNS.

Image analysis settings

Acetylcholine receptors quantification

9. Acetylcholine receptors localization/quantification was performed in the ROI muscle using the "*Find Spots*" module on the α-bungarotoxin channel [**Figure 2C**(1)]. A total of two distinct methods were used to extract the properties of the AChR spots: method C, more sensitive to the detection of the exact number, and method D, more sensitive to the detection of the area. It is also possible to extract raw data on the number of AChR clusters (method C) and their area (method D) in px^2 or μm^2, as well as their average labeling intensity (see **Supplementary Figure 1**).

Axonal length

10. Using the "*Find Image Region*" module only in the surrounding region (body without the CNS), we created a new ROI: axons without the CNS, corresponding to the axonal region without the spinal cord [**Figure 2C**(2)]. This is particularly important because the bright spinal cord signal hampers the detection of weaker axonal staining. With this restricted region, we applied a "*Modify Population*" module with "*Cluster by Distance*" (2 px distance and area > 300 px^2) to individualize axons. An axon selection step can be added to eliminate detection problems with the "*Selection Population*" module (in case some axons have not been properly individualized).

11. Finally, we used the "*Calculate Morphology Properties*" module to measure individual axonal length. The data are presented as length units (μm) for length and width and μm^2 for axonal area (see **Supplementary Figure 1**).

Overlap neuromuscular junction

12. To measure AChR/axon overlap, we used the "*Calculate Position Properties - Cross Population*" module to obtain the overlap percentage between the AChR total area and axon ROI [**Figure 2C**(3)]. This calculation corresponds to the percentage of the AChR cluster area included in the axon ROI area compared to the total AChR area. It is built on the raw data of the axon ROI area (μm^2) and the postsynaptic AChR area (μm^2) calculated using the method D (see **Supplementary Figure 1**). To enable the reproducibility of data and use of the analysis sequences obtained by the Harmony software (Perkin Elmer), all the pre-prints are added in HTML format as **Supplementary Datasheet 1**.

- Validation of this methodology (**Figure 2D**): We validated this novel methodology with the zebrafish model of giant axonal neuropathy. GAN is a fatal disease, presenting a widespread phenotype which starts with a peripheral neuropathy in infancy and extends to the brain in young adults (Lescouzères and Bomont, 2020) (**Figure 1B** and **Table 1**). Since we identified gigaxonin as the defective protein in this disease (Bomont et al., 2000), we have generated a robust zebrafish model of the pathology by (1) transiently repressing gigaxonin expression using morpholino antisense oligonucleotides and (2) creating a knockout line. Both zebrafish models reproduce the loss of motility described in GAN patients (Arribat et al., 2019). Using our high-resolution and quantitative imaging methodology, we refined the role of gigaxonin in controlling (1) AChR clustering, (2) axonal outgrowth/projection, and (3) stability of the NMJ. In addition, the combination of this novel high-content image analysis pipeline with high-throughput screening allowed us to pinpoint drugs capable of restoring all three parameters and develop a therapeutic scheme for preclinical studies (Lescouzères et al., Submitted).
- Advantages of the method: One of the main advantages of this approach is the generation of standardized and quantifiable data for parameters that are usually assessed with either qualitative measures or manual and tedious quantitative tools. In particular, the intention is to address the challenge of quantitative analysis of the NMJ with high throughput. Here, the analysis is automated and allows for robust characterization of a novel phenotype and/or the testing of a large number of compounds in drug screening. Moreover, since the data are obtained from a large number of larvae, it is possible to collect more robust statistics in different populations (diseased/treated larvae, between different genes, etc.). For diseased embryos with modest or less penetrant phenotype, the statistical power of such analysis is extremely high. In addition, our methodology enables the establishment of thresholds from which a specific NMJ-associated parameter is considered affected and/or restored in the treated diseased embryos. Thus, this novel imaging methodology designed for the neuromuscular system presents various advantages that only require a precise positioning of the zebrafish embryos in wells. Noteworthy, the imaging methodology we propose has no time limit and may, in principle, allow the observation of early events of the neuromuscular contacts. To study MN differentiation, (1) the first wave of primary motor neurons at the end of gastrulation (9–10 hpf) or (2) the second wave of secondary motor neurons (14–15 hpf) can be imaged using islet staining (Eisen et al., 1986; Westerfield et al., 1986). Prior to the establishment of

motor nerve terminal contacts, postsynaptic muscle fibers form primitive, dynamic, and non-synaptic AChR clusters that are distributed on the membrane of the adaxial cells. This step, called muscle pre-patterning (Panzer et al., 2005; Wu et al., 2010), can be specifically investigated with the α-bungarotoxin staining between 14 and 16 hpf. In addition, axonal branching and outgrowth are studied at more advanced stages, that is, 26–30 hpf for the pMNs (CaP, MiP, and RoP) and 30–50 hpf for sMNs. (Myers, 1985) using znp1 and zn8 staining, respectively. Finally, this workflow for high-throughput image analysis could be easily adapted to study other tissues, such as the retina in the zebrafish. This adaptation would only require the selection of sufficiently specific antibodies, to ensure the reproducible detection thresholds from one embryo to another.

Conclusion and future prospects

The zebrafish species shares great genetic similarities with humans and is a model of choice for studying the physiology of the neuromuscular system. On the one hand, in the fundamental field, the access to all embryological stages and the ease of use of the model (imaging and housing) allow for the integration of the zebrafish in many comparative studies and the identification of key molecular players in the neuromuscular system. In the field of human genetics, the zebrafish has proven its strength for diagnosis purpose, gene identification, and modeling diseases of the motor neuron, NMJ, and muscle. Taking advantage of the small size and optic transparency of the embryo/larva, behavioral and cellular methodologies uncovered fundamental and disease mechanisms sustaining motor functions. Still, limitations lie in the strength of the assays as one could argue that all NMDs or molecular players look similar, with altered locomotion and decreased axonal outgrowth. Increasing precision is not only necessary to fully decipher the molecular pathways controlling the neuromuscular unit but also mandatory to differentiate diseases and offer the specificity required for personalized medicine. While behavioral assays have been enriched to quantify various parameters of motility in an automated manner, current imaging protocols do not reach this level of development. Here, we present a novel, standardized, automated, and quantitative imaging pipeline for the key parameters of the neuromuscular unit, including the so far challenging quantification of the NMJ. As validated in our GAN disease model, we expect our imaging and analysis tools to be broadly useful to researchers interested in scrutinizing the motor system in detail and executing high-throughput screening in zebrafish models of NMDs.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

This animal study was reviewed and approved by the French ministry (reference N°036) for the creation of the gan zebrafish line.

Author contributions

PB contributed to the conception and design of the work, drafting the manuscript and figures, and critical revisions of the manuscript. LL and BB generated and designed the figures. LL executed the drawing of figures. All authors performed the bibliography search and wrote and reviewed the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2022.956582/full#supplementary-material

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