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Publication Date

2018-07-01

DOI

10.1016/j.neuro.2018.04.016

Peer reviewed

NeuroToxicology 67 (2018) 102–111

Contents lists available at ScienceDirect

NeuroToxicology

Review

Opportunities and challenges for using the zebrafish to study neuronal connectivity as an endpoint of developmental neurotoxicity

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ARTICLE INFO

Article history:
Received 14 February 2018
Received in revised form 23 April 2018
Accepted 24 April 2018
Available online 25 April 2018

Keywords:
Axons
Dendrites
Developmental neurotoxicity (DNT)
In vivo imaging
Neuronal connectivity
Screening platform
Synapses
Zebrafish

ABSTRACT

Chemical exposures have been implicated as environmental risk factors that interact with genetic susceptibilities to influence individual risk for complex neurodevelopmental disorders, including autism spectrum disorder, schizophrenia, attention deficit hyperactivity disorder and intellectual disabilities. Altered patterns of neuronal connectivity represent a convergent mechanism of pathogenesis for these and other neurodevelopmental disorders, and growing evidence suggests that chemicals can interfere with specific signaling pathways that regulate the development of neuronal connections. There is, therefore, a growing interest in developing screening platforms to identify chemicals that alter neuronal connectivity. Cell-cell, cell-matrix interactions and systemic influences are known to be important in defining neuronal connectivity in the developing brain, thus, a systems-based model offers significant advantages over cell-based models for screening chemicals for effects on neuronal connectivity. The embryonic zebrafish represents a vertebrate model amenable to higher throughput chemical screening that has proven useful in characterizing conserved mechanisms of neurodevelopment. Moreover, the zebrafish is readily amenable to gene editing to integrate genetic susceptibilities. Although use of the zebrafish model in toxicity testing has increased in recent years, the diverse tools available for imaging structural differences in the developing zebrafish brain have not been widely applied to studies of the influence of gene by environment interactions on neuronal connectivity in the developing zebrafish brain. Here, we discuss tools available for imaging of neuronal connectivity in the developing zebrafish, review what has been published in this regard, and suggest a path forward for applying this information to developmental neurotoxicity testing.

Contents

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https://doi.org/10.1016/j.neuro.2018.04.016
0161-813X/

1. Introduction

There is increasing consensus that environmental factors interact with genetic susceptibilities to determine the risk and/or severity of diverse neurodevelopmental disorders, ranging from autism spectrum disorders (ASD) to attention deficit hyperactivity disorder (ADHD) to intellectual disabilities to schizophrenia (Lein, 2015; Lyall et al., 2017; Mandy and Lai, 2016). While diverse environmental factors have been implicated in the pathogenesis of neurodevelopmental disorders, much attention has focused on the human chemosphere, including chemical contaminants in the environment, chemicals in personal care products, food additives, and drugs. However, the identity of specific chemicals that influence the risk and/or severity of neurodevelopmental disorders, and the mechanism(s) by which they interact with genetic susceptibilities to contribute to adverse neurodevelopmental outcomes remain outstanding data gaps. There are compelling reasons to identify chemical risk factors for neurodevelopmental disorders. Notably, because in contrast to genetic risks, which are currently irreversible, environmental factors are modifiable risk factors. Therefore, identifying specific environmental factors that increase risk for neurodevelopmental disorders may provide rational approaches for the primary prevention of the symptoms associated with these disorders.

Genetic, histologic, *in vivo* imaging and functional data are converging on altered patterns of neuronal connectivity as the biological basis underlying the behavioral and cognitive abnormalities associated with many neurodevelopmental disorders and intellectual disabilities (Bourgeron, 2009; Garey, 2010, Geschwind and Levitt, 2007; Penzes et al., 2011; Rubenstein and Merzenich, 2003; Svitkina et al., 2010). The candidate genes most strongly implicated in the causation of neurodevelopmental disorders encode proteins that regulate the patterning of neuronal networks during development and influence the balance of excitatory to inhibitory synapses (Belmonte and Bourgeron, 2006; Bourgeron, 2009; Delorme et al., 2013; Stamou et al., 2013). Neuronal connectivity refers to the structural and chemical interactions that connect neurons to form a functional circuit. Critical determinants of neuronal connectivity include the number, length and branching patterns of axons and dendrites, which constitute the neuron's primary site of efferent output and afferent input, respectively, as well as the formation and stabilization of excitatory and inhibitory synapses (Chiu et al., 2014; Lein, 2015; Stamou et al., 2013). These findings suggest that screening for chemicals that interfere with axonal growth, dendritic arborization or synapse formation/stabilization is a feasible approach for identifying potential chemical risk factors for neurodevelopmental disorders, and for elucidating the mechanisms by which chemicals interact with genetic susceptibilities.

Chemical effects on neuronal connectivity have been reported using primary neuronal cell culture, typically derived from developing rodents (Bal-Price et al., 2010; Chen et al., 2017; Sethi et al., 2017; Wayman et al., 2012a, b), and more recently, using neural precursor cells derived from human induced pluripotent stem cell (iPSC) cultures (Druwe et al., 2015; Ryan et al., 2016). While human iPSC-derived neuronal cell models may better predict the effects of neurotoxic chemicals on the developing human brain than primary rodent neuronal cell culture (Ryan et al., 2016), human iPSC-derived neuronal cell cultures present challenges in that they do not yet reliably differentiate into mature neurons with distinguishable axons and dendrites and functional synapses. Regardless of species, *in vitro* models do not fully recapitulate the complex cell-cell and cell-matrix interactions or systemic influences known to influence development in the vertebrate nervous system (Lein et al., 2005). Moreover, it is difficult to correlate changes in neuronal connectivity with deficits in behavior (Lein et al., 2005). *In vivo* developmental neurotoxicity (DNT) studies, predominantly using rodent models, have also been used to demonstrate changes in dendritic complexity and brain morphology due to developmental toxicant exposure (Wayman et al., 2012b; Yang et al., 2009). However, *in vivo* rodent studies are time- and cost-prohibitive for screening, and gene editing can be challenging (Bal-Price et al., 2012, 2010; Lein et al., 2005). Embryonic zebrafish overcome many of the challenges associated with *in vitro* models and traditional *in vivo* rodent models. The inherent advantages of this model coupled with recent advances in imaging provide a powerful approach for *in vivo* studies of chemical effects on neuronal connectivity in the developing nervous system. Thus, the goal of this review is to present the toolbox that could be adapted to study neuronal connectivity in the developing nervous system of the larval zebrafish, and to review published examples illustrating their use.

2. Embryonic zebrafish as a model system for studying neuronal connectivity

The zebrafish has become a powerful research tool in the field of developmental neurobiology and developmental neurotoxicology (Brady et al., 2016; Garcia et al., 2016; Kalueff et al., 2016; Lein et al., 2005; Nishimura et al., 2016; Patton and Zon, 2001; Wiley et al., 2017). Inherent advantages of the zebrafish model include optical transparency, *ex utero* development, which eliminates confounds associated with maternal toxicity, rapid nervous system development, significantly lower costs than traditional *in vivo* rodent models, and adaptability for higher throughput screening compared to rodent models. Zebrafish express homologs for >70% of human genes (Howe et al., 2013), and their genome is fully sequenced (http://www.ensembl.org/Danio_rerio/Info/Index), which facilitates genetic manipulations for both short- (transient) and long-term (stable) transgene and mutant expression. This genetic tractability enables not only mechanistic studies, but also integration of relevant human gene mutations into screens of gene by environment interactions.

An important consideration in the context of DNT is that the fundamental mechanisms of neurodevelopment are highly conserved between zebrafish, humans and other vertebrate models (Gilbert, 2010). Like mammals, zebrafish have a three-part brain structure (telencephalon, mesencephalon and rhombencephalon). Zebrafish and mammals have similar mechanisms of early developmental patterning (homeobox gene gradients etc.) and cellular differentiation/proliferation (radial glial cells, etc.), and they express a similar range of neurochemical phenotypes, including GABA (Higashijima et al., 2004), glutamate (Higashijima et al., 2004), serotonin (McLean and Fetcho, 2004), dopamine (McLean and Fetcho, 2004), norepinephrine (McLean and Fetcho, 2004), glycine (Higashijima et al., 2004) and acetylcholine (Panula et al., 2010). Zebrafish also possess all of the classical sense modalities, including vision, olfaction, taste, touch, balance, and

hearing; and their sensory pathways share an overall homology with humans.

Developmentally, there is one major structural difference between mammalian and zebrafish neurodevelopment: during early neurulation, the zebrafish neural tube is characterized by eversion, whereas the mammalian neural tube undergoes evagination (Wullimann and Mueller, 2004; Wullimann and Rink, 2002). Therefore, zebrafish brain structures are inverted relative to rodents and humans, with the ventricle forming around the outside of the brain instead of internally. While this changes the placement of brain structures in the zebrafish brain relative to the mammalian brain, other aspects, including the mRNA expression patterns, and cell proliferative zones, remain synonymous with that of mammalian brains (Mueller and Wullimann, 2005). Of note, forebrain structures are conserved between zebrafish and mammalian models: the zebrafish subpallium is homologous to the mammalian basal ganglia (Aoki et al., 2013; Mueller et al., 2008); the dorsal and ventral pallium are synonymous with the hippocampus and amygdala, respectively (Mueller et al., 2011; Rodriguez et al., 2002; Wullimann, 2009); and the habenula is homologous to the dorsal diencephalon (Hendricks and Jesuthasan, 2007a). Cognitive behavioral tests suggest that anatomic substrates of cognitive behavior are also conserved between fish and other vertebrates. Thus, similar to observations of hippocampal lesions in mammals, lesions of the structural homolog of the hippocampus in fish selectively impair spatial memory (Rodriguez et al., 2002).

Because of the homologies between zebrafish and mammalian neurodevelopment, zebrafish have been used extensively to study mechanisms of neurodevelopment (Chapouton et al., 2010; Wullimann, 2009; Wullimann and Mueller, 2004) and, more recently, to model human neurodevelopmental disorders (Hoffman et al., 2016; Ijaz and Hoffman, 2016; Meshalkina et al., 2017; Noyes et al., 2015; Stewart et al., 2014). Zebrafish are also being increasingly used for DNT studies. The endpoints of DNT often assessed in zebrafish studies include gross teratological deficits, transcriptional changes, and behavioral readouts (Chueh et al., 2016; Levin and Tanguay, 2011; Mandrell et al., 2012; Truong et al., 2011, 2014). Notably absent from DNT studies in zebrafish are outcomes focused on structural changes in neuronal connectivity, particularly in the central nervous system (CNS). Relevant endpoints of neuronal connectivity that have been examined in the developing zebrafish nervous system include outgrowth of axons and dendrites, synapse formation and synaptic activity (Fig. 1). Below, we review the tools currently available for *ex vivo* and *in vivo* imaging techniques that can be leveraged to quantify neuronal connectivity in developing zebrafish, and we discuss their relative advantages and disadvantages (Table 1).

3. *Ex vivo* imaging techniques

Two *ex vivo* techniques commonly used in other model organisms –*in situ* hybridization to localize RNA and immunohistochemistry to localize protein antigens – have been successfully adapted for use in embryonic and larval zebrafish. Both techniques require that the embryos or larvae be fixed, thus, dynamic changes in neuronal connectivity are difficult to capture. Of the two techniques, *in situ* hybridization is used more widely in zebrafish because of the higher specificity and relative ease of designing probes specific for target RNA. The Thisse lab has developed a library of *in situ* hybridization data from zebrafish larvae, including many neural-specific targets (Thisse and Thisse, 2004). These data, and the probes used to generate them, are publically available on www.zfin.org. In 2005, Mueller and Wullimann published an in-depth atlas of early zebrafish brain development using *in situ* probes to identify transcriptomic profiles in specific brain regions and cell types (Mueller and Wullimann, 2005). Despite a wealth of tools and information, there are some drawbacks of using RNA *in situ* hybridization to obtain details of structural changes during DNT. The gene expression information obtained using standard *in situ* hybridization protocols is often of lower resolution than other labeling techniques. Moreover, because mRNA translation often takes place proximal to the cell body, *in situ* hybridization typically does not enable visualization of structural determinants of neuronal connectivity, e.g., axons, dendrites and synaptic contacts

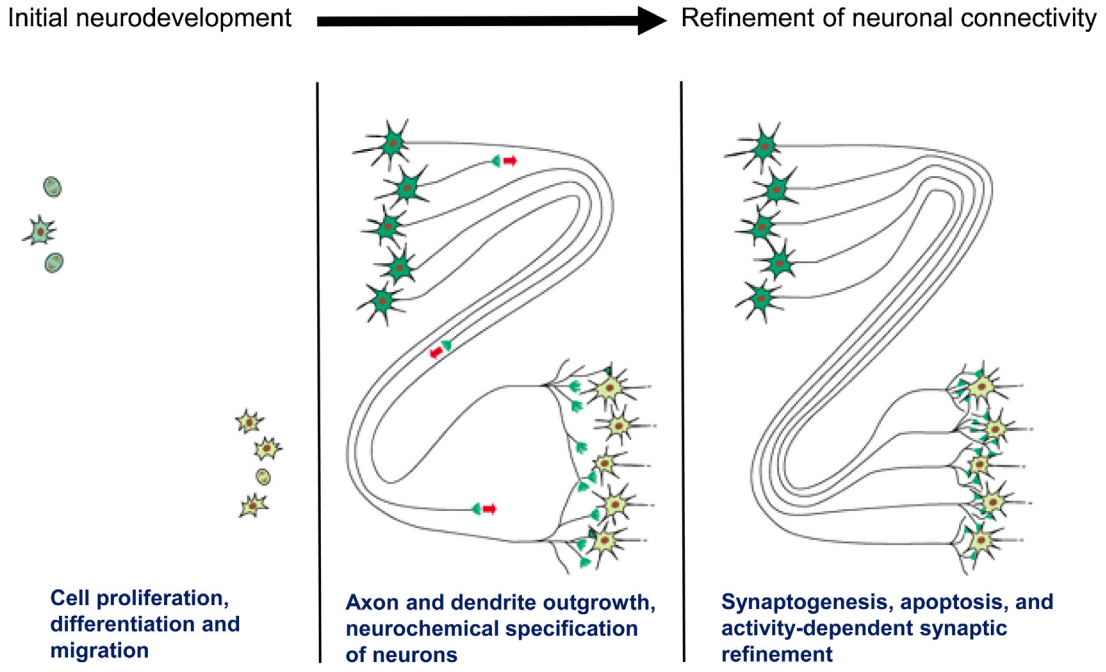

Fig. 1. Schematic illustrating the aspects of neuronal connectivity that have been imaged in the developing zebrafish.

Table 1
Techniques for neuronal imaging in larval zebrafish.

Approach	Whole mount	Strain	*In/Ex vivo*	Time to implementation	Consistency	Advantages	Disadvantages
Immunohisto chemistry (IHC)	Yes	Any	*Ex vivo*	<1 week from sampling	Good	Whole mount or sections, validated antibodies	Limited cross- species reactivity for zebrafish proteins with available antibodies
RNA *in situ* hybridization	Yes	Any	*Ex vivo*	<1 week from sampling	Good	Large body of comparable work, whole mount or sections, highly specific targeting	Targets RNA not protein (does not account for post-translational modifications)
Array tomography	No	Any	*Ex vivo*	<1 week from sampling	Good	High-resolution imaging of IHC, ISH or genetic labeling techniques	Increased processing of both samples and images
Transgenic lines: Enhancer trap FLIP trap Brainbow, etc.	Yes	Specific to target	*In vivo*	6+ months	Good	Stable and reliable expression patterns, fluorescent labels allow for high resolution imaging	Relatively long time to create, need separate lines for examining different expression patterns
Injection and/or electroporation for expression of transient genetic markers	Yes	Any	*In vivo*	Immediate	Variable	Mosaic expression, rapid implementation, possibility of single cell labeling	High levels of mortality, mosaic expression, invasive, inter sample variation in labeling
Dyes/indicators	Yes	Any	*In vivo*	Immediate	Variable	Rapid implementation, mosaic expression, possibility of single cell labeling	High levels of mortality, invasive, inter-sample variation in labeling

(Wu et al., 2016; Yoon et al., 2016). These disadvantages may be overcome by combining RNA *in situ* hybridization with immunohistochemistry.

Immunohistochemistry has been used both in whole-mount (Fig. 2) and sectioned larval zebrafish preparations to examine specific subsets of neurons in the tail, optic tectum and areas of the brain (Easley-Neal et al., 2013; Yang et al., 2011). There are numerous validated, zebrafish-specific, neural-targeted antibodies, many of which are available through, or described on, the Zebrafish International Resource Center (ZIRC) website (http://zebrafish.org). However, in comparison to mammalian models, immunohistochemistry has been limited in zebrafish embryos due to poor cross reactivity of antibodies developed against mammalian antigens with zebrafish antigens. As newer antibodies are developed specifically against zebrafish antigens, it is likely that immunohistochemistry will become increasingly valuable for studying neural connectivity in the developing zebrafish nervous system.

Array tomography, which combines immunohistochemistry, plasticized ultra-thin sections and high-resolution three-dimensional immunofluorescence imaging, could prove to be a viable method for visualizing neural connectivity in zebrafish *ex vivo* (Leung et al., 2013; Micheva et al., 2010; Micheva and Smith, 2007; Robles et al., 2011; Wang and Smith, 2012). This approach enhances image resolution, thereby allowing for collection of information regarding neuronal connectivity and other structural changes to the nervous system (Robles et al., 2011). The main drawbacks of using array tomography include resource requirements (equipment and trained personnel), and increased time required to process both samples and images.

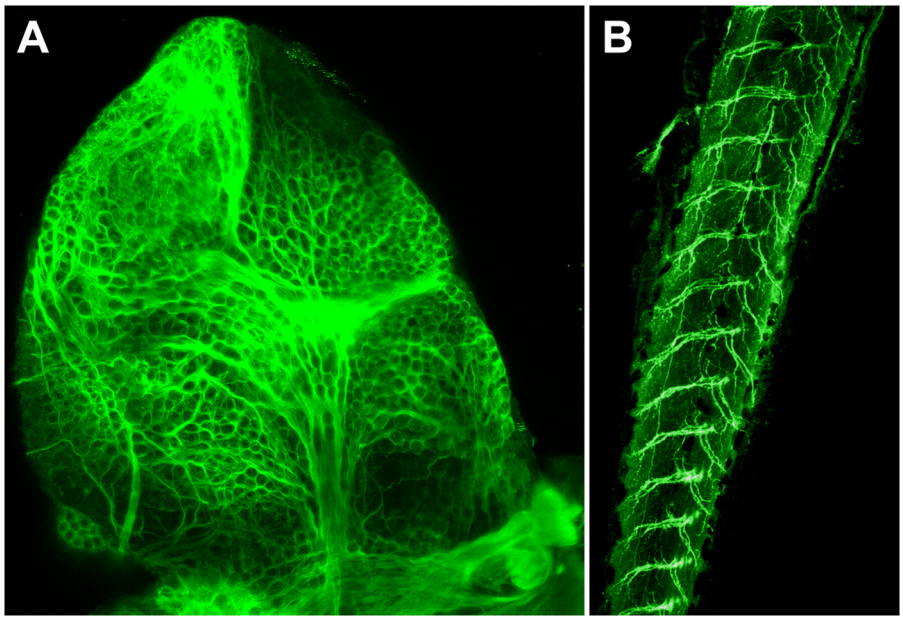

Fig. 2. Immunohistochemical staining of zebrafish larvae using an antibody against acetylated tubulin. Zebrafish larvae at 5 days post-fertilization were fixed and stained with an antibody against acetylated tubulin, and then imaged using a Leica STED confocal microscope. Representative photomicrographs illustrate tubulin immunoreactivity in the brain (A) and tail (B) of zebrafish larvae.

3.1. Examples illustrating the use of ex vivo imaging to assess neuronal connectivity in developing zebrafish

In general, most published work using *ex vivo* imaging has focused not on visualizing neuronal cell morphology or structural parameters of neuronal connectivity, but rather on identifying neuronal cell types (Higashijima et al., 2004; McLean and Fetcho, 2004) and brain regions (Mueller et al., 2011, 2008; Wullimann and Rink, 2002) in the developing zebrafish brain. A notable exception is a study that combined fluorescence *in situ* hybridization, immunohistochemistry and genetic markers with array tomography to identify and characterize GABAergic cells in the tectum and periventricular neurons (Robles et al., 2011). Additionally, *ex vivo* imaging techniques have been employed to study effects of chemicals on: (*i*) motor neuron malformation (Babin et al., 2014; Svoboda et al., 2002; Welsh et al., 2009; Yang et al., 2011); (*ii*) ganglion cell, Schwann cell and oligodendrocyte patterning (Parng et al., 2007); (*iii*) axonal patterning in the brain and spinal cord (Li et al., 2009); and (*iv*) developmental RNA expression patterns in the optic nerve (Roy et al., 2016).

4. *In vivo* imaging techniques

Many *in vivo* genetic and imaging tools have been developed to study the genetic regulation of zebrafish nervous system development. These genetic tools are used in conjunction with advanced imaging approaches, such as confocal microscopy or light sheet microscopy (Fig. 3), and computational methods to examine the structure and function of the developing zebrafish nervous system. In recent years, light sheet microscopy has become a valuable tool for visualizing not only morphological processes, but also neuronal activity in the whole brain of embryonic zebrafish. Advantages of light sheet microscopy include its low embryonic phototoxicity, high signal-to-noise ratio, as well as high spatiotemporal resolution, which is necessary for visualizing fast morphological events with high resolution [reviewed in (Ahrens et al., 2013; Icha et al., 2016; Keller and Ahrens, 2015; Panier et al., 2013)]. These tools and techniques are adaptable for DNT studies of chemical effects on neuronal connectivity, as discussed in this section.

4.1. Direct dye labeling

Lipophilic carbocyanine dye tracing (DiI, DiD, DiO, etc.) has been successfully used for anterograde and retrograde neuronal cell labeling in zebrafish (Jontes et al., 2000; Zou et al., 2014). These dyes diffuse along the lipid membrane of injected cells, resulting in isolated whole-cell labeling. They are versatile and can be used in both live and fixed tissues. Although carbocyanine dyes allow for visualization of cellular morphology, labeling is not precise, and inter-sample variation is common. Similarly, neuronal activity can be measured by injecting calcium-sensing dyes into the brain of live fish (Kassing et al., 2013; Niell and Smith, 2005). While this technique allows live Ca^{2+} imaging, it is quite invasive, sample to sample variation is common and acute toxicity and/or trauma is a concern. Thus, for detecting significant differences between experimental groups, a large sample size is often needed. Because of this and the labor-intensive nature of these procedures, these techniques may be useful for mechanistic studies, but currently are not feasible for medium throughput chemical screening applications.

4.2. Genetic tools for in vivo imaging

A large number of cDNA plasmids and transgenic fish lines have been generated to examine the morphology of diverse populations of neurons by fluorescence. Enhancer trap screening and bacterial artificial chromosome transgenesis, sometimes in combination with the Tol2 transposon system, have been used to create fluorescent tagged enhancer trap lines that show pan-neuronal expression or expression limited to neuronal cell subpopulations (Asakawa and Kawakami, 2008; Asakawa et al., 2008; Satou et al., 2013; Scott and Baier, 2009; Suster et al., 2009; Yoshida et al., 2010). The Tol2 transposable element has been used to generate multiple enhancer trap and gene trap constructs, which are included in the zTrap database (http://kawakami.lab.nig.ac.jp/ztrap/) (Kawakami et al., 2010). This database provides a useful resource for identifying fish lines that have the desired embryonic patterns for evaluations of neurotoxicologic effects of chemicals.

Many of the enhancer trap lines use green fluorescent protein (GFP) as their fluorophore, which limits visualization of the interactions between different subsets of neurons. This limitation has been addressed by generating transgenic fish lines that enable combinatorial gene expression strategies. Two of the most commonly used combinatorial expression strategies are: (*i*) binary systems such as GAL4-UAS, Lex A and Tet systems; and (*ii*) recombination-based systems such as Cre/loxP or Flp/FRT systems. These two strategies are reviewed in greater detail elsewhere (Hocking et al., 2013, Scott, 2009, Weber and Koster, 2013). Briefly,

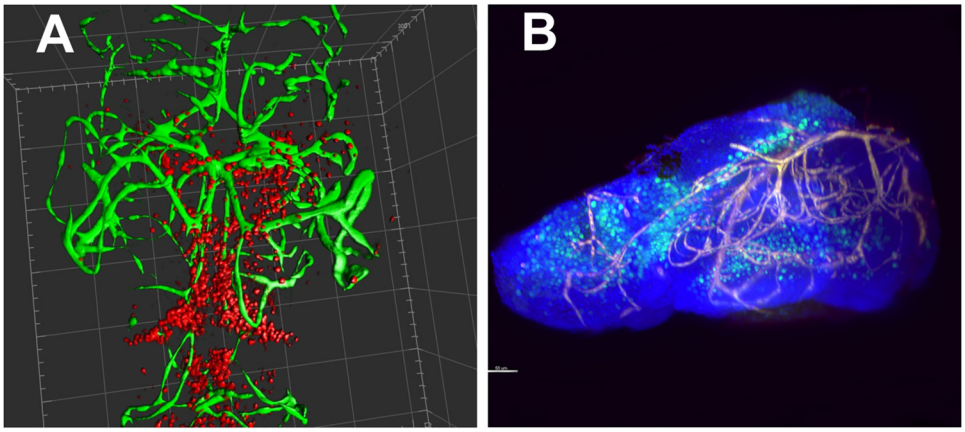

Fig. 3. Representative images obtained using light sheet microscopy of transgenic zebrafish expressing fluorescent proteins linked to neural-specific promoters. (A) Dorsal view of brain illustrating GABAergic neurons (red) and blood vessels (green). (B) Lateral view of brain showing GABAergic neurons (green), blood vessels (yellow) and cell nuclei (blue). Embryos were fixed at 120 h post-fertilization, brains extracted and then stained overnight with 4′,6-diamidino-2-phenylindole (DAPI). Brains were then mounted in capillaries with 1.5% low-melt agarose and optically cleared in 2,2′-thiodiethanol (TDE) before imaging with ZEISS Lightsheet Z.1 at 20X magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

in the GAL4-UAS binary system, instead of coupling the enhancer region or a minimal promoter to GFP, the promoter or enhancer element is placed upstream of the GAL4 gene. This ensures GAL4 protein expression in the tissue of interest. Once the GAL4 protein is expressed, the GAL4 binds to the UAS sequence and drives the expression of any gene downstream of the UAS sequence. Many transgenic lines expressing these binary systems are listed in the Zebrafish International Resource Center (ZIRC) database (http://zebrafish.org).

Of the many publications to date describing the use of the Gal4/UAS driver system in the zebrafish, one of the most influential utilized the yeast Gal4-DNA binding domain fused to the herpes simplex virus transcriptional activation domain VP16 (Gal4-VP16) to increase expression of the UAS-driven target (Koster and Fraser, 2001). The Gal4-VP16 system significantly amplifies transgene expression, and can drive the expression of multiple target genes. This has proven to be a valuable resource for *in vivo* imaging. Over 150 transgenic enhancer trap lines and their expression patterns are listed in a study by Scott and Baier, with expression patterns described in many specific regions of the brain and spinal cord (Scott and Baier, 2009). Similarly, approximately 100 Gal4 enhancer trap lines that show brain-specific expression have recently been identified, and these are publicly available via ZIRC (Otsuna et al., 2015). Using a combination of Gal4-VP16 and Cre-Lox with bacterial artificial chromosome recombineering, 22 Gal4- and Cre- based drivers containing upstream and downstream gene regulatory regions for cell-type specific neurotransmitters, neurotransmitter synthesizing enzymes or neuropeptides were generated with each of the transgenic lines labeling a specific subset of neurons (Forster et al., 2017). In addition, the FLIPtrap lines created and maintained by the Caltech Center for Excellence in Genomic Sciences provide additional fluorescently tagged enhancer trap lines with genes expression in the zebrafish nervous system (http://fliptrap.usc.edu/static/anatomies.html). These enhancer trap zebrafish lines provide a valuable resource for examining the effects of chemical exposure on a specific neuronal population. It is important to note that some of the available GAL4 lines are maintained as frozen sperm, which may require 6+ months prior lead time to generate larval zebrafish for mechanistic studies or screening applications.

One of the drawbacks of the enhancer trapping strategy is that many of these lines have non-neuronal expression in addition to expression in the nervous system, which sometimes limits their utility for studying cellular morphology of developing neurons. The use of photoconvertible reporters, such as UAS-Kaede, allows examination of specific neurons/neuronal processes in a population of labeled cells (Scott et al., 2007). Incorporation of the neuron restrictive silencing element in the transgene has been demonstrated to limit non-neuronal expression in transgenic lines, thereby making these transgenic zebrafish extremely valuable for examining neuronal morphology (Bergeron et al., 2012). A recent imaging study (Marquart et al., 2015) of about 100 GAL4, Cre and enhancer trap lines showed that using UAS–reporter transgenes with 3′UTR sequences that bound non-neuronal microRNAs significantly limited non-neuronal expression of these transgenes. This makes many of the known GAL4 lines more useful for analysis of neuronal cell populations. The expression patterns of these transgenes are available on a 3-D database of gene expression known as Brain Browser (https://science.nichd.nih.gov/confluence/display/burgess/Brain+Browser), as well as the zebrafish information network (Zfin, http://zfin.org) and the zebrafish enhancer trap database maintained by the Burgess lab (http://burgesslab.nichd.nih.gov/).

One of the most exciting advances of these combinatorial systems for visualizing neurons has been the generation of the multi-labeled Zebrabow/Brainbow fish lines and plasmids (Pan et al., 2013). These fish express a DNA construct that encodes for three fluorophores – red fluorescent protein (RFP), cyan-fluorescent protein (CFP) and yellow fluorescent protein (YFP) – under the control of a ubiquitous promoter or multiple copies of UAS, with each fluorophore flanked by two unique LoxP sites. The UAS-Zebrabow constructs can be expressed in distinct subsets of neurons by using tissue specific GAL4 drivers. Under basal conditions, the first fluorophore (RFP) in the construct is expressed in all cells. When Cre recombinase is introduced (either as an injected mRNA/protein or by crossing to a Cre-transgenic line), the fluorophores are stochastically expressed throughout the affected cell types due to the activity of the Cre recombinase on the flanking LoxP sites. The end effect is a range of colors observed throughout target tissues with neighboring cells often displaying distinct colors (Pan et al., 2013, 2011; Weber and Koster, 2013). Thus, these lines provide an excellent method for imaging not only the neuronal morphology of individual cells within a population, but also the interactions between connecting neurons.

Mosaic expression of reporter constructs can also be generated by injecting cDNA plasmids into the embryo. Early stage (1–2 cells) injection with plasmids encoding Brainbow fluorophores (driven by a CMV promoter since neuron-specific Thy promoters do not work in zebrafish) into Cre-transgenic lines, or co-injection of Cre-recombinase mRNA/protein into other lines, results in mosaic expression of the fluorophores, thus allowing for non-specific tissue expression in any fish line (Pan et al., 2013). Alternatively, plasmid/mRNA can be injected directly into the third or fourth ventricle at 24 h post-fertilization. If these injections are closely followed by electroporation across the brain, the plasmids will be incorporated into the developing neurons (Cerda et al., 2006; Dong et al., 2011; Hendricks and Jesuthasan, 2007b; Tawk et al., 2009). Injecting at 24 h post-fertilization catches neurons during proliferation (radial glia, neuronal stem cells, etc.), and facilitates labeling of neurons in different regions of the brain.

4.3. Examples illustrating the use of in vivo imaging to assess neuronal connectivity in developing zebrafish (summarized in Table 2)

4.3.1. Labeling of dendrites in the CNS

Dendritic arbor formation is a dynamic process and critical structural determinant of neuronal connectivity. Neurotoxic chemicals have been shown to interfere with normal patterns of dendritic arborization, and altered dendritic complexity is linked to multiple neurodevelopmental disorders (Copf, 2016; Lein, 2015; Stamou et al., 2013). Imaging of complete dendritic arbors in the intact zebrafish brain is difficult, but tools have been developed to make these measurements possible. For example, dendritic arborization and synapse formation has been quantified in the optic tectum of developing zebrafish by injecting a GAL4/UAS plasmid (Niell et al., 2004). The plasmid used for these studies contained a pan-neuronal goldfish *alpha-1 tubulin* driver (Koster and Fraser, 2001), and expressed both dsRed, a whole cell biomarker, and GFP-tagged PSD95, a postsynaptic marker (Niell et al., 2004). Using a membrane-targeted yellow fluorescent protein driven by the *aldoca* (aldolase c, fructose-biphosphate a) promoter, neuronal cell polarization and dendrite formation of Purkinje cells was visualized in the zebrafish cerebellum (Tanabe et al., 2010). Using membrane-bound fluorescent proteins, dendrite formation in retinal ganglion cells was imaged using two-photon confocal microscopy (Choi et al., 2010). Similarly, several populations of retinal ganglion cells labeled with separate fluorophores driven by cell-type specific promoters have been imaged (Mumm et al., 2006). Another study used a combination of membrane targeted Brainbow with bacterial artificial chromosome transgenic lines using DsRed and GFP to label glycinergic and glutamatergic neurons in the hindbrain of zebrafish larvae

Table 2
Examples of live imaging.

Target	Method	Brain region or cell type	Reference
Dendrites	Transgenic lines controlled by *isl2b* or *brn3c* RGC specific promoters and transient expression driven by a Gal4/UAS system controlled by the same promoters	RGCs	(Mumm et al., 2006)
	Transgenic lines expressing membrane bound fluorescent proteins driven by either *isl2b* or *brn3c* RGC specific promoters	RGCs	(Choi et al., 2010)
	Membrane-targeted yellow fluorescent protein driven by the Purkinje cell specific *aldoca* (aldolase c, fructose-biphosphate a) promoter	Purkinje cells	(Tanabe et al., 2010)
Dendrites and synaptic connections	GAL4 driver controlled by the pan-neuronal goldfish *alpha-1 tubulin* promoter and UAS activator expresses dsRed (whole cell) and PSD95:GFP (presynaptic marker)	Tectum	(Niell et al., 2004)
Synaptic connections	Live staining using DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine) and DiO (3,3'-dioctadecyloxacarbocyanine perchlorate)	Spinal neurons	(Jontes et al., 2000)
	Marked presynaptic terminals using GFP-linked N-cadherin, and verified by counterstaining for the synaptic vesicle protein SV2	Spinal neurons (RB)	(Jontes et al., 2004)
	GFP-Synaptophysin and cytosolic DsRed, both driven by a Gal4/UAS system and targeted using an RGC-specific *brn3C* promoter	Optic tectum and RGCs	(Meyer and Smith, 2006)
	Transgenic line neurogenin1:GFP and fluorescently tagged synaptic proteins using the Gal4/UAS system driven by a RB neuron specific promoter	Spinal neurons (RB)	(Easley-Neal et al., 2013)
Synaptic activity	Calcium indicator dye directly injected into the tectal neuropil	Tectum	(Niell and Smith, 2005)
	The genetically encoded calcium indicator (GECI) transgenic lines under control of various neuronal promoters	Various	(Akerboom et al., 2012)

RGC = retinal ganglion cells.
RB = Rohon Beard cells.

(Kinkhabwala et al., 2011). This approach allowed visualization of not only the unique morphologies of glycinergic *vs*. glutamatergic neurons, but also their physical associations to each other. While these studies focused on the polarization, initiation and formation of dendritic arbors, the techniques used could be readily applied to assess the effects of chemicals on dendritic complexity throughout the CNS.

4.3.2. Synaptic markers

Neuronal synapses are specialized cell-cell junctions that allow communication between neurons. The distribution and processing of information in the nervous system is determined by the pattern of synaptic connections formed between neurons during development. Imbalances in the types and number of synapses formed during development can contribute to adverse clinical outcomes. When visualizing synaptic connections, it is important to mark both the presynaptic cell (i.e. the axon) and the postsynaptic cell (i.e. the dendrite or soma), as these connections, especially during development, are dynamic. While pre- and postsynaptic molecules are conserved in the zebrafish genome (Bayes et al., 2017), relatively few of these have been targeted for *in vivo* imaging. Of the available studies, the predominant focus has been on presynaptic markers. Axonal growth cones and early synaptogenesis have been visualized using the dyes DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine) and DiO (3,3′-dioctadecyloxacarbocyanine perchlorate) to mark growing neurons in the zebrafish spinal cord (Jontes et al., 2000). The same group later identified presynaptic terminals using GFP-linked *N*-cadherin and verified these data by counterstaining for the synaptic vesicle protein SV2 (Jontes et al., 2004). Expression of the fusion protein was driven in neuronal tissue using a Gal4/UAS system (Jontes et al., 2004; Koster and Fraser, 2001). A combination of GFP-synaptophysin and cytosolic DsRed (both driven by a Gal4/UAS system) was used to demonstrate the dynamic process of synaptic formation and stabilization in the developing zebrafish nervous system (Meyer and Smith, 2006), and fluorescent markers have been used to study synapsin trafficking during synapse formation in spinal neurons (Easley-Neal et al., 2013). While many of these techniques were developed to determine the kinetics of synapse formation in zebrafish spinal neurons, they could be easily repurposed for use in the zebrafish brain, and for studying the effects of chemicals on synapse formation and number in the developing brain.

4.4. Synaptic activity measurement

The small size of the zebrafish embryonic brain, coupled with the linear organization of the brain and the inversion of brain structure (i.e. the “deeper” neurons are on the surface) enables imaging of activity within the whole brain using current microscopy techniques (Feierstein et al., 2015; Leung et al., 2013). Injection of a calcium indicator dye into the tectal neuropil has been used to analyze visual responses in live larval zebrafish (Niell and Smith, 2005). A similar, non-invasive method for measuring brain activity involves the use of genetically encoded Ca^{2+} indicators (GECIs). The GECI lines, when driven by pan-neuronal promoters and imaged using two photon microscopy or light sheet microscopy, allow visualization of synaptic activity, even at the level of single action potential within the developing zebrafish brain (Akerboom et al., 2012). The newer generation calcium sensors have been used to measure activity in the whole zebrafish brain, and in response to specific behavioral traits such as optomotor behavior and fictive behavior in larval zebrafish (Ahrens et al., 2012; Randlett et al., 2015). In addition, immunohistochemical staining for extracellular signal–regulated kinase (ERK) was used as another readout for neuronal activity (Randlett et al., 2015). These data were used to generate a zebrafish brain atlas (Z-brain - http://engertlab.fas.harvard.edu/Z-Brain/) that correlates neuronal activity and neuroanatomical information. GECIs in combination with advanced imaging and processing techniques can even allow for Ca^{2+} imaging in freely swimming larval fish (Cong et al., 2017; Kim et al., 2017; Knafo et al., 2017).

5. Conclusions

The larval zebrafish provides an unparalleled opportunity for medium throughput *in vivo* neuronal imaging of structural and functional parameters of neuronal connectivity. Both *ex vivo* and *in vivo* techniques for imaging neuronal connectivity at the cellular level have been developed for the developing zebrafish brain but have yet to be applied to DNT research. Additionally, new visualization tools, microscopy methods and computational tools

Table 3
Zebrafish resources for gene expression information.

Database	Description of available tools	Website link
zTrap	Fish Enhancer trap and gene trap lines with diverse embryonic expression patterns	http://kawakami.lab.nig.ac.jp/ztrap
ZIRC	Zebrafish International Resource Center website has information about fish lines such as GAL4 lines, EST/cDNAs, Antibodies etc.	http://zebrafish.org
Brain Browser	3-D database with gene expression patterns for around 100 enhancer trap lines with neuronal expression patterns.	https://science.nichd.nih.gov/confluence/display/burgess/Brain+Browser
ZFIN	Zebrafish model organism database containing fish lines, gene expression data, antibodies etc.	http://zfin.org
Zebrafish Enhancer Trap Database	The database contains information for the neuronal enhancer trap lines generated by the Burgess lab	http://burgesslab.nichd.nih.gov/
FLIPTRAP	Database of existing FLIP trap alleles, some of which have neuronal gene expression	http://fliptrap.usc.edu/static/anatomies.html

are continuously being developed across diverse model organisms that could be adapted to the zebrafish [reviewed by (Lee et al., 2016)]. *Ex vivo* techniques are a good starting point, since generating or acquiring transgenic lines is time consuming (Table 1). This approach is facilitated by the availability of extensive reviews describing resources available to study the nervous system of zebrafish larvae [reviewed by (Chapouton et al., 2010); also see Table 3. Using these techniques, researchers can identify affected regions/cell types and find/create appropriate transgenic lines. Alternatively, mosaic expression (e.g. transient genetic markers) under the control of a pan-neuronal driver can help identify DNT effects in specific brain regions.

Incorporating these imaging techniques will require modifications to increase throughput and reproducibility. In our experience, use of available non-stable transgenic markers produces unreliable expression patterns and is not currently practical for DNT screening applications. Additionally, these techniques are often equipment intensive, requiring sophisticated and expensive microscopes and image analysis tools to obtain and process the data generated. These problems are surmountable and the addition of neuronal morphogenic data to the extensive databases of genetic, “omic”, teratological and behavioral phenotypic data already published will be a significant advance for understanding DNT and gene by environment interactions that contribute to the pathogenesis of neurodevelopmental disorders.

Conflict of interest

None.

Acknowledgements

We thank Ingrid Brust-Mascher and Colin Reardon of the UC Davis Health Science District Advanced Imaging Facility for assistance in generating the representative photomicrographs shown in Figs. 2 and 3. This work was supported by the National Institute of Environmental Health Sciences (grants ES014901, ES011269 and ES023513) and the United States Environmental Protection Agency (grant R833292). GWM was supported by a postdoctoral fellowship from the National Institute of Environmental Health Sciences (F32 ES024070).

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