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***Xenopus laevis* as a model system to study cytoskeletal dynamics during axon pathfinding**

Paula G Slater, Laurie Hayrapetian, and Laura Anne Lowery

Department of Biology, Boston College, Chestnut Hill, Massachusetts

Abstract

The model system, *Xenopus laevis*, has been used in innumerable research studies and has contributed to the understanding of multiple cytoskeletal components, including actin, microtubules, and neurofilaments, during axon pathfinding. *Xenopus* developmental stages have been widely characterized, and the *Xenopus* genome has been sequenced, allowing gene expression modifications through exogenous molecules. *Xenopus* cell cultures are ideal for long periods of live imaging because they are easily obtained and maintained, and they do not require special culture conditions. In addition, *Xenopus* have relatively large growth cones, compared to other vertebrates, thus providing a suitable system for imaging cytoskeletal components. Therefore, *Xenopus laevis* is an ideal model organism in which to study cytoskeletal dynamics during axon pathfinding.

Keywords

microtubule dynamics; cytoskeleton; growth cone

Introduction

During neural development, neurons must elongate their axons to form new connections and migrate towards their final destination. The dynamic structure in charge of axon navigation is the growth cone, located at the tip of the axon and rich in cytoskeletal components. The growth cone is exposed to morphogen gradients and guidance cues that activate internal signaling cascades, thereby generating changes in actin filament (F-actin) and microtubule (MT) dynamics. Growth cone protrusion and retraction are tightly regulated by the balance of F-actin polymerization, retrograde flow, and F-actin severing (Dent *et al.*, 2011; Lin *et al.*, 1996; Lin and Forscher, 1995; Mallavarapu and Mitchison, 1999; Okabe and Hirokawa, 1991). MTs also play a significant role in growth cone pathfinding: stable MTs in the proximal domain steer growth cone advance, and dynamic MTs entering the distal region of the growth cone act as guidance sensors (Holy and Leibler, 1994; Tanaka *et al.*, 1995). As a result of cytoskeletal regulation, the growth cone can undergo morphological changes that control axon advance, turning, and retraction (Dickson, 2002; Gomez and Letourneau, 2014; Lowery and Van Vactor, 2009).

Despite decades of research into axon guidance, there is still much to learn regarding cytoskeletal coordination downstream of different signaling cues. To study cytoskeletal regulation, multiple complementary experimental strategies are required; of these, live imaging may be one of the most vital (Schnell *et al.*, 2012). Therefore, it is crucial to have a suitable model system in order to enhance our understanding of growth cone cytoskeletal dynamics. For more than 60 years, *Xenopus* has been used as a model organism to study aspects of early vertebrate development, including neuronal migration and axonal pathfinding. We and others have found that *Xenopus laevis* (*X. laevis*) is an excellent model system for studying cytoskeletal dynamics during axon pathfinding, growth cone motility, and axon guidance. In addition, *Xenopus* is also an ideal model for studying the cytoskeleton in a broad number of cell migration contexts. In this mini-review, we compare *X. laevis* to other model systems and highlight the importance of using *Xenopus* to study cytoskeletal dynamics during axon pathfinding.

1. Advantages of *Xenopus laevis* as a model organism

Many model systems have been widely used to study axon guidance and development, including hippocampal neurons from rat and mouse embryos (Andersen and Bi, 2000), bag cell neurons from adult *Aplysia californica* (Lee *et al.*, 2008; Suter and Miller, 2011), dorsal root ganglia neurons from chicken embryos (Dontchev and Letourneau, 2003; Fantetti and Fekete, 2011), and retinal ganglia cells and spinal cord neurons from *X. laevis* embryos (Chien and Harris, 1994; Erdogan *et al.*, 2016; Santiago-Medina *et al.*, 2011). While all systems have specific advantages and disadvantages, it is clear that *X. laevis* provides an ideal system for studying the role of the cytoskeleton, because it has neuronal growth cones that are easy to obtain, manipulate, culture, and image at a low cost (Fig. 1).

Xenopus genetics—Recent advances in *Xenopus* genetics and genomics have expanded the tools available for using *Xenopus* as a model system. To date, twenty species from the *Xenopus* genus have been identified. Among them, two have been widely used as model organisms: *Xenopus laevis* and *Xenopus tropicalis*. The genomes of both have been recently sequenced and have displayed high gene collinearity with the human genome (Hellsten *et al.*, 2010; Session *et al.*, 2016). *X. laevis*, in particular, has been well-utilized to study axon guidance and development, due to their significantly larger growth cone size (Erdogan *et al.*, 2016).

In order to support *Xenopus* research, two resources have been created: The NIH *Xenopus* Initiative and Xenbase. The NIH *Xenopus* Initiative includes expressed sequence tags (EST sequences and UniGene Clusters), full-length cDNA sequences, cDNA libraries, genomic libraries, mutagenesis and phenotyping projects, and *X. tropicalis* genome sequencing (Klein *et al.*, 2002). Meanwhile, Xenbase recompiles literature and annotated genetic information to organize the *Xenopus* community knowledge base. It also provides useful sequence services, such as the genome browser and *Xenopus* specific blast tool (Bowes *et al.*, 2008). In addition, central repository/stock centers for *Xenopus*, such as the National *Xenopus* Resource, also exist. The National *Xenopus* Resource provides a facility for breeding *X. laevis* and *X. tropicalis*, maintaining genetic stocks, supplying stocks to researchers, and developing new experimental tools and husbandry techniques (Pearl *et al.*, 2012). These

resources can be used to expand genetic knowledge and study cytoskeletal dynamics during axon pathfinding in *Xenopus* growth cones.

Culturing *Xenopus laevis*—There are multiple advantages that *X. laevis* has over other organisms in terms of the logistics of neuronal culturing, including the shorter timing for obtaining cultures, the ability to provide large numbers of eggs, the *ex utero* fertilization, and the easy-to-maintain neuronal cultures.

X. laevis cultures are obtained at a faster rate than other model systems (Fantetti and Fekete, 2011; Kaech and Banker, 2006; Lee *et al.*, 2008; Lowery *et al.*, 2012). *X. laevis* retinal ganglion cells and neural tube explants are dissected from stage 28 and 20 embryos respectively, approximately one day after fertilization. Meanwhile, in chicken, the dorsal root ganglion neurons are obtained from 6-day old embryos (Fantetti and Fekete, 2011). Also, bag cell neurons are obtained from adult *Aplysia californica* (Lee *et al.*, 2008), which has a 43-day development time (Havenhand, 1993). In addition, rat and mouse hippocampal primary neuronal cultures are prepared from 16–18 days old embryos (Kaech and Banker, 2006).

To obtain embryos from *X. laevis*, female frogs can be stimulated to lay several thousands of eggs simply by a priming injection of chorionic gonadotropin hormone (Fig.1), 12–18 hr before eggs are needed. After obtaining eggs, fertilization can occur *ex utero* with minced testes. *X. laevis* neural tube explants can then be obtained 24 hr after the fertilization process (Fig.1), sending out axons which can be examined 12–24 hr later (Lowery *et al.*, 2012). While *X. laevis* can be stimulated to lay eggs multiple times in a lifespan, rodent pregnant dams must be sacrificed in order to obtain embryos. Additionally, *X. laevis* can lay a larger number of eggs than many other organisms, allowing more embryos per collection than any other model system mentioned herein.

X. laevis fertilization *ex utero* enables researchers to study and manipulate embryos in any desirable stage of development, and the quick development of *X. laevis* embryos allows for rapid analysis (Keller, 1991). Furthermore, *X. laevis* neurons can be maintained easily at room temperature without requiring CO₂ level regulators (Lowery *et al.*, 2012), like *Aplysia californica* (Lee *et al.*, 2008). In contrast to *X. laevis*, rat and mouse cultures require incubation to regulate both temperature and CO₂ levels (Kaech and Banker, 2006).

Genetic manipulation—In order to study the contribution of a specific gene to axon guidance and development, gene expression can be modified with delivery of RNA or antibody treatments to suppress gene products, or exogenous mRNAs or DNA, which allow overexpression of wildtype and mutant proteins. Cytoskeletal dynamics can then be studied by evaluating the effect of the specific manipulated gene in the corresponding process.

Numerous techniques enable rapid *X. laevis* gene manipulation, providing advantages to *X. laevis* as a model system over other vertebrate embryos. *X. laevis* embryos develop *ex utero*, they are large enough to manipulate with minimal training, their stages of development have been characterized (Nieuwkoop and Faber, 1994), and the fate of the cells comprising the blastomeres have been extensively studied (Moody, 1987a, 1987b). The most common

technique used to manipulate gene expression in *X. laevis* is microinjection of mRNA or DNA for over-expression (Gomez *et al.*, 2003; Lowery *et al.*, 2013; Nwagbara *et al.*, 2014), as well as antisense morpholino oligonucleotides for protein knockdown (Blum *et al.*, 2015; Lowery *et al.*, 2013; Nwagbara *et al.*, 2014). However, like many knockdown strategies, morpholinos can sometimes produce off-target effects. Although the use of standard control morpholinos can ameliorate these effects (Eisen and Smith, 2008), more recent alternatives for genetic knockdown have also been developed, including Transcription activator-like effector nucleases (TALENs) (Ken-ichi *et al.*, 2013; Nakajima and Yaoita, 2015; Sakane *et al.*, 2014) and CRISPR-Cas9 (Guo *et al.*, 2014). Electroporation has also been widely used to manipulate gene expression. Electroporation can be applied to multiple or single cell organisms using selective promoters, which allow for cell-specific expression. In *X. laevis*, targeted injections and subsequent electroporation can be used to spatially and temporally control gene expression in later embryonic stages (Bestman *et al.*, 2006; Eide *et al.*, 2000).

In order to manipulate gene expression in whole rats and mice, CRISPR-Cas9 in germlines (Chapman *et al.*, 2015) and embryo electroporation *in utero* (Tabata and Nakajima, 2001) have been used. While both methods have produced significant results, they have slower generation times and require an experienced investigator. Electroporation and transfection of hippocampal neurons have also been used widely, but it is limited by the low efficiency of transfection (1%–10%) (Kaech and Banker, 2006). Microinjection of DNA (Kaang, 1996) and mRNA (Sahly *et al.*, 2003) in cultured neurons from *Aplysia*, as well as electroporation in chicken embryos (Momose *et al.*, 1999), are other examples of limited gene transfer techniques in different organisms.

Xenopus laevis imaging—The growth cone is the cytoskeleton-rich dynamic structure that guides axon pathfinding and elongation. To study cytoskeletal dynamics during axon pathfinding, a major focus of research has been centered on the growth cone. Therefore, one of the most important features to consider when choosing a model system for studying cytoskeletal dynamics in axon pathfinding is the size of the growth cone. The model systems, zebrafish *Danio rerio* and *Xenopus tropicalis*, provide many of the advantages listed above (ease of external development, genetic manipulation, neuronal culturing); however, both organisms have significantly smaller growth cones than *X. laevis*, which preclude cytoskeletal analysis. Rat hippocampal neurons in cultures extend growth cones of 5–10 μm in diameter (Paglini *et al.*, 1998); dorsal root ganglion neurons from chicken embryos extend even smaller growth cones than rat hippocampal neurons (Tosney and Landmesser, 1985); *Xenopus laevis* extend a larger growth cone, 10–30 μm in diameter (Lowery *et al.*, 2012). Growth cones from the mollusk, *Aplysia californica*, can be prompted to expand to sizes 5–10 times larger than vertebrate neurons (Lee *et al.*, 2008), allowing striking imaging of cytoskeletal dynamics. However, under these conditions, the net advancement of this large and over-stabilized state of *Aplysia californica* growth cones can be compromised (Ren and Suter, 2016), limiting the capacity to observe outgrowth, turning, or retraction events. Thus, *Xenopus laevis* provides a larger and easier-to-culture growth cone than any other vertebrate model system.

When studying the dynamic structure of the cytoskeleton, it is crucial to have a model system suitable for imaging protein localization in both fixed samples and live cells at high

temporal and spatial resolution. Live imaging offers several advantages over fixed imaging. Fixed samples require specific and low-background antibodies. Furthermore, some low-expressed proteins and/or fluorescent dyes are lost during the fixation process. Proteins tagged with fluorescent dyes and observed during live imaging bypass this issue (Santiago-Medina *et al.*, 2011; Schnell *et al.*, 2012). Most importantly, live imaging allows one to observe and understand changes in growth cone motility and associated cytoskeleton changes over time, providing more extensive information than fixed images. Since *X. laevis* neurons are cultured at room temperature, live imaging can be performed for long periods of time without the need of regulated temperatures and CO₂ imaging chambers. The same is true for *Aplysia californica*. But for rat, mouse and chicken neurons, imaging chambers are indispensable.

The cross-talk between MT and F-actin that guides growth cone steering is necessary for axon pathfinding (Zhou and Cohan, 2004). For this reason, F-actin (Burkel *et al.*, 2007; Petchprayoon *et al.*, 2005) and MT associated proteins (Honnappa *et al.*, 2009; Lowery *et al.*, 2013; Nwagbara *et al.*, 2014; Stepanova *et al.*, 2003) have been widely used as probes to study cytoskeletal dynamics. Because of its small diameter and high packing density, F - actin is beneath the optical resolution limit. Therefore, super resolution microscopy or electron microscopy must be used to study the precise F-actin spatial organization and interactions with MTs (Engel, 2014; Xu *et al.*, 2012). This technique has been used successfully to study cytoskeletal components in *X. laevis* (Marx *et al.*, 2013).

Additionally, several software packages can greatly facilitate quantitative analysis of cytoskeletal dynamics. PlusTipTracker (Applegate *et al.*, 2011) allows tracking of MT plus-ends to analyze MT dynamics. Similarly, Quantitative Fluorescent Speckle Microscopy (QFSM) (Mendoza *et al.*, 2012) can be used to track the movement and turnover kinetics of macromolecules within the cell. Both types of software packages have been used successfully to study MT and actin dynamics in *X. laevis* growth cones (Lowery *et al.*, 2013; Stout *et al.*, 2014).

2. Contribution of *Xenopus laevis* to the study of cytoskeletal dynamics during axon pathfinding

Research utilizing *X. laevis* growth cones has already contributed significant insights to our knowledge of different cytoskeletal components in axonal outgrowth. Several studies in recent years are highlighted here.

Xenopus laevis neural tubes and retinal cultures have been used to study the role of the actin cytoskeleton during growth cone advancement. A critical aspect of axonal outgrowth is the adhesion of the growth cone to the extracellular matrix (ECM), achieved by point contacts that bind the ECM to the actin cytoskeleton through adhesion proteins. Woo and Gomez (2006) demonstrated that coordinated Rho GTPase activity is necessary for the generation of point contacts, which stimulate neurite growth and dynamics (Woo and Gomez, 2006). In addition, Cdc42 (Myers *et al.*, 2012) and the Rac1/Cdc42 guanine nucleotide exchange factor Vav2 (Moon and Gomez, 2010) are needed to promote neurite formation and branching. Moreover, Nichol *et al.* (2016) showed that a negative correlation exists between retrograde F-actin flow and rapid migration of growth cones with comparatively more point

contacts (Nichol *et al.*, 2016). While point contacts bind growth cones to ECMs, infiltrating cells need a more stable source for ECM contact, such as the invadosome. Santiago et al (2011) showed that growth cones extend F-actin-rich invadosomes, containing metalloproteinases to promote ECM remodeling and proper axon extension (Santiago-Medina *et al.*, 2011).

MTs are tightly regulated by different MT associated proteins. The proteins that regulate MT plus-end dynamics are called plus-end-tracking proteins (+TIPs). These proteins inform and direct growth cone behavior (Bearce *et al.*, 2015; Cammarata *et al.*, 2016). *Xenopus* has been used as a model to study +TIPs and their effects on MT dynamics in the growth cone. Using high-resolution live-imaging in cultured *Xenopus* growth cones, it was discovered that TACC1 (Lucaj *et al.*, 2015), TACC2 (Rutherford *et al.*, 2016) and TACC3 (Nwagbara *et al.*, 2014) can all act as +TIPs and positively regulate MT growth velocities. Lowery et al (2013) determined that XMAP215 is necessary for persistent axonal outgrowth, and that it may affect MT plus-end velocities in growth cones by regulating MT and F-actin coupling (Lowery *et al.*, 2013). In addition, Marx et al (2013) used *Xenopus* neurons cultured from embryonic neural tube explants to demonstrate that the +TIP protein XCLASP1 positively modulates MT advances and promotes axon extension (Marx *et al.*, 2013).

The cytoskeletal component that has been studied less than actin or MTs is the neurofilament, which is important for later stages of axonal development. Experiments involving neuronal cultures of *X. laevis* embryonic neural tube explants have been used to study the effect of perturbing neurofilaments on axonal outgrowth. Undamatla et al (2001) showed that neurofilaments of different subunit compositions have differential expression timing and localization within neurites (Undamatla and Szaro, 2001). Additionally, developing axons lacking neurofilaments are shorter than the control ones (Lin and Szaro, 1995). These axons lacking neurofilaments grow more slowly because they spend smaller fractions of their growth cycles extending than normal axons from the same *Xenopus* embryos (Walker *et al.*, 2001). Studies have also shown that neurofilaments are present in long stabilized branching, but not in shorter ones, suggesting that neurofilaments participate in axonal branching stabilization during neuronal development (Smith *et al.*, 2006).

Conclusion

Each of the model systems used to study axon guidance and development have their own unique advantages. With these organisms, many contributions have been made to advance cytoskeletal studies. However, it appears that no other model organism has growth cones as large, easy, and inexpensive to culture, manipulate or image as *X. laevis*. Cumulatively, these factors provide substantial support to consider *X. laevis* as one of the best vertebrate model systems available for studying the cytoskeleton in axon guidance and development.

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Figure 1. Obtaining *Xenopus* embryonic explants

Cartoon of the *Xenopus* embryonic explants dissection, culturing and imaging. CG chorionic gonadotropin.