Topic Introduction

In Vivo Imaging of Synaptogenesis in Zebrafish

James D. Jontes and Michelle R. Emond

The embryonic zebrafish is a nearly ideal model system in which to use time-lapse imaging to study the development of the vertebrate nervous system *in vivo*. The embryos are small and transparent, they develop externally and rapidly, and the embryonic central nervous system is relatively simple and highly stereotyped. With the refinement of green fluorescent protein (GFP) as a genetically encoded fluorescent tag of neuronal proteins, along with advances in imaging technology, it is possible to follow the cellular and molecular events underlying development as they occur in the living embryo. This article describes strategies for imaging synapse formation in the embryonic zebrafish.

INTRODUCTION

The chemical synapse is a specialized cell–cell junction that mediates polarized interneuronal communication through regulated exocytosis. Each neuron can form hundreds or thousands of presynaptic terminals onto other neurons, as well as receiving hundreds or thousands of synaptic inputs. These synapses often form at sites distant from the neuronal cell body along extensively arborized axons or dendrites. The function of the nervous system, to a great extent, is governed by the patterns of synaptic connectivity established during development. However, the mechanisms involved in synapse formation remain incompletely understood.

What are the steps involved in assembling the synaptic junction? How are the constituent synaptic proteins recruited to nascent synapses? How are these components, integral and peripheral membrane proteins, as well as soluble proteins, delivered into axons and dendrites to be made available for synaptic assembly? How is the spatial distribution of synaptic components within the synapse organized? Is there a requisite temporal sequence to assembling this organization? What mechanisms govern the selection of appropriate synaptic partners, thereby determining the time and place of junction formation?

Addressing these questions experimentally can be difficult. Synaptogenesis is an inherently dynamic process with both synapse formation and elimination occurring asynchronously as ongoing, concurrent processes. Adequately understanding synaptogenesis requires both temporally and spatially resolved information of cellular and molecular dynamics. In addition, synaptogenesis is critically dependent on context; it requires specific cell–cell interactions that occur at particular times and places within a well-defined developmental framework. Thus, attempts at reducing the inherent complexity of the experimental system could result in arbitrary behavior and misleading results.

An ideal approach to studying synaptogenesis would involve preserving spatial and temporal information, as well as the developmental and structural aspects of the system under investigation. With the simultaneous development of vital dyes and fluorescent proteins, as well as confocal and multiphoton microscopy, it is becoming possible to investigate the molecular and cellular mechanisms of synaptogenesis in a variety of organisms *in vivo*. In particular, the embryonic zebrafish is a nearly

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ideal system for imaging synaptogenesis in an intact embryo: (1) The zebrafish is a vertebrate; (2) embryos develop externally and rapidly; (3) embryos are small and transparent; (4) the early nervous system is relatively simple and highly stereotyped; (5) there are straightforward ways of reliably expressing fluorescent transgenes in specific neuronal populations; and (6) the zebrafish is amenable to both forward and reverse genetic approaches. In this article, we describe some general considerations for imaging synaptogenesis in the intact zebrafish embryo. A detailed protocol is also available; see **Fluorescence Imaging of Transgenic Zebrafish Embryos** (Jontes and Emond 2012).

FLUORESCENT TAGGING OF SYNAPTIC PROTEINS

Fusion Proteins

A synaptic molecule fused to GFP or another color variant is a prerequisite for imaging synaptogenesis using time-lapse microscopy. Relatively few GFP–fusion proteins have been developed for use in zebrafish. Presynaptically, these include VAMP/synaptobrevin (Jontes et al. 2004), synaptophysin (Meyer and Smith 2006), Rab3 (Campbell et al. 2007), N-cadherin (Jontes et al. 2004), and protocadherin- α (Emond and Jontes 2008). However, sequences for all of the most common presynaptic molecules are present in the databases, and fusion proteins can easily be generated in a week or two. These include active zone molecules (syntaxin-1, SNAP-25, CAST/ERC, CASK, Piccolo, and Bassoon), vesicle-associated molecules (synapsins, SV2s, VGluts), and putative adhesion molecules (SynCAMs, cadherins, neuroligins, nectins, and sidekicks). Thus, an increasing variety of options should become available.

The choice of fusion protein will reflect the specific goals of the study. For example, the most commonly used presynaptic markers *in vitro* and *in vivo* label synaptic vesicles (VAMP, synaptophysin, and synapsin) and yield a punctate fluorescence signal indicating the presence of synaptic vesicle clusters. However, such markers might not be the most reliable for capturing the earliest events in synaptogenesis, which could precede synaptic vesicle clustering. In addition, the use of synaptic vesicle markers needs to be interpreted carefully. For example, an impairment in vesicle clustering will be indistinguishable from a defect in synapse formation. In such cases, an active zone marker, such as CAST/ERC or Bassoon, might be a better choice to report on the presence of presynaptic terminals. Finally, not all presynaptic proteins are punctate and therefore will not function as synaptic labels. In our hands, CASK-GFP labels axons diffusely (J.D. Jontes and M.R. Emond, unpubl.), and VAMP-GFP shows a considerable background labeling of the axonal plasma membrane. As for presynaptic fusion proteins, the choice of synaptic marker will depend on the goals of the specific experiments. Figure 1 presents a gallery of common presynaptic GFP–fusions.

At present, the only published postsynaptic GFP–fusion protein in zebrafish is PSD95-GFP (Niell et al. 2004). N-cadherin-GFP is also postsynaptic, but has not yet been used as a postsynaptic marker. Again, most of the common postsynaptic molecules have either been cloned (e.g., the NMDA receptor and AMPA receptor subunits; Cox et al. 2005; Hoppmann et al. 2008) or are easily accessible through database searches (e.g., neuroligins).

As with all GFP–fusion proteins, it is essential to validate the distribution and behavior of synaptic markers. It is important to determine that GFP–fusion puncta actually represent synaptic contacts. In some circumstances, it can be important to verify that the GFP–fusion accurately reflects the distribution of the endogenous protein; however, this might not be necessary if the fusion protein is only being used as a synaptic marker. The most common method for validating synaptic localization is to use immunocytochemistry to colocalize the fusion protein with antibodies against well-known synaptic markers. Ideally, a presynaptic fusion protein should appose with antibodies against a postsynaptic protein, and vice versa. At present, colocalization by immunocytochemistry presents two major difficulties in zebrafish. First, there are relatively few antibodies against synaptic proteins that work in zebrafish. The most commonly used is a monoclonal antibody against the synaptic vesicle protein, SV2. Reliable antibodies against postsynaptic proteins are not yet widely available. However, this is likely to change rapidly as more researchers generate antibodies against zebrafish synaptic proteins.

FIGURE 1. Expression of presynaptic markers. Shown here are spinal axons expressing GFP or presynaptically localized GFP–fusion proteins. Soluble GFP acts as a space-filling marker of neuronal morphology, but can also appear punctate as it accumulates in axonal varicosities and branchpoints in axonal and dendritic arbors. VAMP/synaptobrevin-GFP and synaptophysin-GFP label synaptic vesicles and vesicle precursor organelles. Like VAMP or synaptophysin, N-cadherin and protocadherin1 γ are transported in intracellular organelles, but differ in that they label the presynaptic plasma membrane. The VAMP-, synaptophysin-, and N-cadherin-GFP fusions were expressed from UAS-driven plasmids, whereas GFP and protocadherin1 γ -GFP were expressed from recombinant BAC clones. Scale bar, 15 μ m.

Second, whole-mount antibody labeling of neuropil is extremely dense. The overlapping signals from adjacent synapses result in images of nearly continuous fluorescence, making colocalization essentially meaningless. In some fortuitous cases, such as early in development when there are fewer neurons or in certain sparsely populated regions of the nervous system, antibody labeling can be used effectively. Labeling of thin or semithin plastic sections might also be a viable alternative.

Promoters/Enhancers

Nearly as important as obtaining an appropriate synaptic marker is obtaining a suitable promoter/enhancer with which to drive the expression of the transgene. Together, the promoter and the fusion protein govern the ability to carry out successful imaging of synaptogenesis in zebrafish embryos. If the type of neuron to be labeled is unimportant, pan-neuronal promoters, such as a 2.7-kb fragment of the goldfish α 1-tubulin promoter (Hieber et al. 1998) or a 3.4-kb sequence upstream of the zebrafish *HuC* gene (Park et al. 2000), might be suitable. Otherwise, there is a growing list of available promoters/enhancers for labeling specific neuronal populations. The goals of the experiment will largely determine the choice of promoter/enhancer.

Gal4-UAS (Upstream Activation Sequence) System

It is a common problem that even a functional promoter will drive gene expression only weakly and inconsistently. Both the mosaicism inherent in transient expression from plasmid injections and the low and variable levels of gene expression can make some experiments impractical. To circumvent these problems, Köster and Fraser (2001) adapted the Gal4-UAS system, widely used in *Drosophila*, for use in transient gene expression in the zebrafish. It is possible to amplify the expression of a transgene by placing Gal4 under the control of a tissue-specific promoter (the driver or activator construct) and by placing the transgene under the control of multiple UAS sites coupled to a minimal promoter (the effector construct). This amplification results in an increase in gene expression levels, as well as a greater number of expressing cells. An additional advantage of Gal4-UAS is that a single driver construct can activate multiple effector cassettes, simultaneously driving expression of multiple transgenes. However, it should be noted that the Gal4-UAS system can be a bit leaky, with spillover expression in skin, muscle, and notochord, particularly during early development.

Bacterial Artificial Chromosomes (BACs)

Short promoter/enhancer elements (generally 1–10 kb of sequence upstream of the transcriptional start site) derived from a target gene are often insufficient to reliably recapitulate the expression

pattern of that gene because required regulatory sequences can be spread over large swaths of genomic sequence, both upstream and downstream of the transcriptional start site. To closely mimic the native expression pattern of a gene, larger genomic regions might be necessary. Over the last several years, a variety of approaches have been implemented for the manipulation of large segments of genomic DNA present in BAC or PAC (P1-derived artificial chromosome) clones. These approaches (i.e., BAC recombineering) are based on homologous recombination and allow seamless insertion, deletion, or modification of DNA sequences (Copeland et al. 2001; Lee et al. 2001; Warming et al. 2005). Recombinant BACs can then be used to generate stable transgenic lines or for transient transgene expression. With the progress being made in the sequencing of the zebrafish genome and the availability of several excellent BAC libraries, it will be possible to express transgenes in nearly any neuronal population.

We regularly employ BAC recombineering to express GFP-tagged proteins using one of three approaches (Fig. 2). Detailed protocols for BAC recombineering can be found at <http://recombineering.ncifcrf.gov/>. The most straightforward approach is to insert a GFP–fusion protein (e.g., synaptophysin–GFP) in place of the first coding exon of a gene that shows an appropriate expression pattern. Ideally, injection of this recombinant BAC should result in the expression of the GFP–fusion in the same population of neurons as the endogenous gene. Similarly, the Gal4 transactivator can be inserted into the BAC, which can be used to drive the expression of any UAS:transgene effector module. Alternatively, if the BAC clone encodes the gene to be studied and is not simply being used to hijack the promoter/enhancer, GFP (or some variant) can be fused in frame with the 5′ or 3′ termini to GFP-tag the gene. This approach can be particularly useful in two circumstances: (1) The gene has multiple splice variants, and the investigator does not want to choose one arbitrarily (e.g., neurexins or clustered protocadherins); and (2) the cDNA for the gene is too large to conveniently clone, tag, and express by conventional approaches (e.g., Piccolo or Bassoon). As is the case with conventional expression constructs, the expression pattern obtained by BAC injections should be verified. Examples of BAC-based transgene expression are shown in Figure 2.

COEXPRESSION OF TRANSGENES

There are numerous circumstances in which it is necessary to coexpress multiple transgenes within a neuron: (1) investigating cotrafficking of proteins or organelles, (2) investigating the relative timing of delivery of two components to a nascent synapse, (3) investigating the effects of overexpression or misexpression of a protein along with a reporter, and (4) investigating the effects of coexpressing dominant-negative or mutant forms of a protein along with a reporter. In *in vitro* systems, this is relatively straightforward. Cotransfection of plasmids is very efficient in cultured neurons, and biolistic approaches can easily introduce multiple transgenes into neurons in slice cultures. However, in zebrafish, the mosaic nature of transgene expression from plasmids makes coexpression more challenging. Simple coinjection of plasmids into 1-cell-stage embryos rarely results in neuronal coexpression, making this approach impractical for regular experimentation.

To coexpress transgenes in zebrafish, we have implemented a bidirectional UAS approach, which relies on the fact that the UAS motif acts as a bidirectional enhancer element (Fig. 3). The core of this expression plasmid is the 14xUAS cassette (Köster and Fraser 2001) flanked by two E1b basal promoters and two multiple cloning sites. Two simple subcloning steps allow reliable coexpression of two transgenes when coinjected with a Gal4 activator plasmid. Although there is some variability in the relative expression levels of the two transgenes, we have observed frequencies of coexpression of >90%.

IDENTIFICATION OF SYNAPTIC PUNCTA

Some synaptic fusion proteins might not behave as anticipated. Most notably, they can appear too diffuse within the neuron and fail to show a punctate distribution. In some cases, this could simply

FIGURE 2. BAC recombineering strategies for expressing transgenes in zebrafish. (A) Four strategies for using BAC recombineering to express GFP or GFP fusions in zebrafish. (1) Structure of a hypothetical gene consisting of six exons, with the ATG start codon internal to exon 1. (2) Expression of GFP by replacing exon 1. (3) Tagging the 3' end of the gene with GFP to generate a carboxy-terminal fusion protein. (4) Tagging the 5' end of the gene with GFP to generate an amino-terminal fusion protein. (5) Driving GFP expression using the Gal4/UAS system. This requires two recombinations. I-SceI sites are shown as arrowheads. (B) Two approaches used for incorporating I-SceI sites into BAC-based expression constructs. (1) Two I-SceI sites are incorporated into the BAC, well downstream from the gene. (2) Recombineering is used to retrieve relevant regions of the BAC clone (15–20 kb) into a modified pBluescript with I-SceI sites. (C) Segment of spinal cord from a 4-dpf (days postfertilization) zebrafish injected with a doubly recombinant BAC clone driving Gal4VP16, but also containing a UAS:GFP effector cassette inserted in a downstream exon. A primary motoneuron and some interneurons can be identified, as well as a series of longitudinal axons. (D) Area of the optic tectum in a 5-dpf zebrafish expressing protocadherin1 α -GFP. GFP was inserted in-frame with the 3' end of the pcdh1 α gene, thus generating a carboxy-terminal GFP–fusion for all 10 of the pcdh1 α isoforms. (E) Retina of a 5-dpf zebrafish expressing synaptophysin-GFP. The BAC clone was modified essentially as shown in Fig. 2A2, but inserting synaptophysin-GFP rather than GFP. (F) Spinal cord of a 4-dpf zebrafish in which a subset of axons is labeled with synaptophysin-GFP. The approach here is similar to that in C, with Gal4 driving UAS:synaptophysin-GFP, which is recombined into the same BAC clone. Scale bars, 10 μ m.

reflect the native distribution for that protein. Some proteins, although known to be synaptic, might not be exclusively synaptic and therefore might not be suitable synaptic markers. Where possible, results should be evaluated against comparable GFP–fusions for that protein in other systems. If a fusion-protein “should” work (i.e., a comparable fusion has been used in another system), the length or composition of the linker segment can be varied, because changing only a few amino acids could change the behavior of the fusion. Both amino-terminal and carboxy-terminal fusions can be considered.

The issue of what constitutes a synapse can be divided into two questions. First, what constitutes a punctum? Second, which puncta represent synapses? With regard to the first question, we set a

FIGURE 3. Coexpression of two transgenes using a bidirectional UAS cassette flanked by multiple cloning sites. After successive subcloning of cDNAs into the left and right sites, coinjection of the dual-gene effector plasmid with a Gal4 driver results in coexpression of the transgenes in the same neurons. For small, easily expressed fusions, coexpression can be seen in >90%–95% of cells. For large or toxic fusion proteins, coexpression can become more variable, manifesting primarily as differences in the relative levels of the two transgenes. This approach is suitable for coexpressing fusion proteins, expressing a fusion protein along with a soluble or membrane-targeted counterstain to visualize cell morphology, or to coexpress a reporter fusion protein with a dominant-negative molecule. (*Left*) Two spinal interneurons in a 2-dpf embryo coexpressing cyan fluorescent protein (CFP) (red) and VAMP-yellow fluorescent protein (YFP) (green). (*Middle*) Axon of a primary motoneuron labeled with Kusabira Orange (red) and synaptophysin-GFP. (*Right*) Region in the spinal cord of a 4-dpf zebrafish expressing Kusabira Orange (red) and PSD95-GFP (green). Scale bars, 10 μ m.

threshold at 2–2.5 times above the mean pixel intensity of diffuse axon staining, then remove detected puncta that are below a size threshold. This process is easily automated either in ImageJ or MATLAB.

With regard to the second question, not all puncta are synapses. Presynaptic components are trafficked within pleiomorphic transport organelles, and postsynaptically, some proteins are found clustered in extrasynaptic puncta. The primary criterion for distinguishing between synapses and transport organelles in time-lapse sequences is the stability of the punctum. Putative synapses are those puncta that, once formed, are stable for the duration of the time lapse. As mentioned above, retrospective immunocytochemistry can be used to determine if stable puncta of the GFP–fusion colocalize with other synaptic markers and can be considered synaptic.

ADVANTAGES AND LIMITATIONS

Imaging synaptogenesis *in vivo* during the development of the zebrafish nervous system offers a number of challenges and potential pitfalls. Theoretically, the quality of the data is not as good as can be obtained by imaging neurons cultured on coverslips. Such preparations can be used with short-working-distance, high-NA (numerical aperture) oil-immersion objective lenses that produce bright, high-resolution images. Also, flat, two-dimensional arrays of neurons can easily be transfected, fixed, and processed for immunolocalization, and are amenable to the use of FM dyes for imaging presynaptic function. Most synaptic molecules have been cloned, and GFP-tagged versions are available, as are well-characterized antibodies. In contrast, apart from a small number of GFP–fusions and an even smaller number of antibodies, most reagents in the zebrafish system have yet to be generated. However, in return for confronting the risks and difficulties associated with studying synaptogenesis *in vivo*, much of the uncertainty faced in *in vitro* studies is eliminated; specifically, the fact that there is no guarantee that events observed *in vitro* faithfully mimic those that occur in an organism during development.

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J.D. Jontes and M.R. Emond

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