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In Vivo Time-Lapse Imaging of Neuronal Development in *Xenopus*

Edward S. Ruthazer, Anne Schohl, Neil Schwartz, Aydin Tavakoli, Marc Tremblay, and Hollis T. Cline

In vivo fluorescence imaging of cells in the developing nervous system is greatly facilitated in specimens in which cells are brightly but sparsely labeled. In this article, we describe a number of techniques that can be used for delivering fluorophore to neurons in the albino *Xenopus laevis* tadpole. Fluorescent dye or DNA that encodes a fluorescent protein can be delivered to single cells by electroporation. Alternatively, multiple cells can be labeled with fluorescent dye introduced by local iontophoresis or with plasmid DNA introduced by bulk electroporation. Technical considerations and analysis methods for time-lapse imaging in living tissue are also discussed.

INTRODUCTION

In vivo time-lapse imaging experiments have revealed mechanisms that modulate branch formation, growth, and stabilization that could never have been surmised from studies in fixed tissue (Kaethner and Stuermer 1992; Balice-Gordon and Lichtman 1993; O'Rourke et al. 1994; Feng et al. 2000; Lendvai et al. 2000; Gan et al. 2003). Collecting accurate time-lapse imaging data in vivo, however, is impeded by factors that do not severely impact experiments in cultured cells. First and foremost is the scattering of light by the overlying tissue. This problem can be considerable when imaging deep brain structures, even in largely transparent animals like *Xenopus* tadpoles or zebrafish, and becomes more severe in adult brain because of increases in myelination, bone calcification, and skin pigmentation. In an effort to improve signal-to-noise ratios, more intense excitation light can be used, but this greatly increases the risk of severe photodynamic cell damage. Another challenge to imaging cells in vivo is the ability to discretely label individual cells or specific populations of cells in the brain structure of interest. Technological advances in microscopy and fluorophore design and delivery, combined with clever strategies for the use of transparent and transgenic animals for imaging studies, have helped overcome many of these problems, bringing in vivo neuronal imaging to prominence in recent years. This article reviews recent technological progress in in vivo time-lapse imaging in *Xenopus*.

LABELING OF NEURONS FOR IN VIVO IMAGING

Fluorescent Dextran

Fluorescent dye-conjugated dextran (3000 MW for retrograde labeling and either 3000 or 10,000 MW for anterograde labeling) is a water-soluble neuronal tracer available in a nearly limitless variety of fluorophore conjugates, ranging from relatively photostable Alexa dyes to calcium reporters such as Oregon Green BAPTA. When used in conjunction with fixable biotinylated dextran amine (which can

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be visualized postmortem in histological sections by incubation with streptavidin-horseradish peroxidase), labeled material can be taken all the way from in vivo fluorescence imaging to the electron microscope (Reiner et al. 2000). The mechanism by which fluorescent dextrans are taken up by neurons is not well understood, but either pressure injection or iontophoretic delivery of a 5% (w/v) dextran solution readily labels many cells (Glover et al. 1986; Schmued et al. 1990). It has been shown that fibers severed during the injection procedure are preferentially labeled (Gahtan and O'Malley 2001). Iontophoresis could therefore increase labeling efficiency by electroporation of the plasma membranes of cells and processes in the vicinity of the ejection micropipette. (Electroporation occurs when micropores in the plasma membrane are induced in an electric field, thereby permitting charged molecules to be driven directly into the cytoplasm.) Images of dextran-labeled neurons can be collected for many days to weeks after labeling without obvious clumping or degradation of the fluorophore, so long as measures are taken to reduce phototoxicity. Thus, fluorescent dextrans might be the most versatile and easily delivered neuronal tracers available. Despite their ease of delivery, or perhaps because of it, it can be difficult to restrict fluorescent dextran labeling to single neurons within a brain structure. However, single-cell electroporation is a relatively simple means to label individual cells with dextrans (see **Labeling Individual Neurons in the Brains of Live *Xenopus* Tadpoles by Electroporation of Dyes or DNA** [Ruthazer et al. 2013a]).

Lipophilic Vital Dyes

Lipophilic fluorescent dyes, which include carbocyanine dyes like DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), and styryl dyes such as DiA (4-(4-(dihexadecylamino)styryl)-*N*-methylpyridinium iodide), are well suited to single-cell labeling (Honig and Hume 1986). Because lipophilic dyes are insoluble in the aqueous environment of the brain or eye, dyes are dissolved in dimethyl sulfoxide, dimethyl formamide, or ethanol and delivered by pressure or iontophoresis through a glass micropipette. They then precipitate instantaneously, resulting in little or no uptake of the dye outside of the small injection site. Using a method adapted from the grasshopper (Myers and Bastiani 1993), juxtacellular iontophoresis under microscopic visualization has been used with considerable success to achieve the targeted delivery of dyes to label single neurons in the brains of living tadpoles. Only cells in contact with the dye crystal will be labeled as the dye diffuses throughout the lipid-rich plasma membrane of the cell, resulting in the sometimes complete labeling of the dendritic and axonal processes of neurons within minutes. Over several days, lipophilic dyes label intracellular membrane compartments in cells, giving a “blebbed” appearance, probably as a result of membrane cycling.

Lipophilic dyes with a fairly wide range of spectral properties and hydrophobicities are available. For in vivo confocal imaging, both DiI and DiO (3,3'-dioctadecyloxycarbocyanine perchlorate) have been found to give satisfactory results, but the long-wavelength carbocyanine dye DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) offers superior labeling with relatively little phototoxicity and tissue autofluorescence at its excitation wavelength of 644 nm and emission peak at 665 nm. Unfortunately, the long excitation wavelengths of DiI and DiD reduce their usefulness for two-photon imaging. On the other hand, DiO and DiA efficiently undergo two-photon excitation at 880 nm, resulting in bright images of neurons and their processes (Ruthazer and Cline 2002).

Genetic Labeling by Fluorescent Protein Expression

The use of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* represents a revolutionary advance in the field of in vivo imaging. The discovery of useful fluorescent proteins from corals (including red variants) and the use of mutagenesis to develop enhanced GFPs (EGFPs) and GFP-like proteins with a broad range of spectral properties has made the GFP family of proteins at least as versatile as extrinsic dyes for most applications (Tsien 1998; Fradkov et al. 2000; Bevis and Glick 2002; Shaner et al. 2004; Miyawaki 2005). Two properties of GFP make it a near-ideal fluorophore for in vivo imaging: The protected fluorophore-in- α - β -barrel structure of GFP renders it unusually resistant to photobleaching and free radical generation, and GFP is efficiently excited by two-photon excitation (Ormö et al. 1996).

For axonal and dendritic tracing, it is necessary to label a small number of cells at a defined site in the brain. In *Xenopus*, DNA can be microinjected into blastomeres or transfected by lipofection into early embryos, resulting in a mosaic of crudely targeted GFP-expressing cells (Ohnuma et al. 2002). Alternatively, this can be accomplished by making stereotaxic injections of virus to target the delivery of DNA encoding GFP to cells at one site in the brain (Wu et al. 1995; Hermens and Verhaagen 1998).

Electroporation of DNA is a convenient alternative method that is becoming increasingly popular. Commonly used to transfect avian embryos, the method now has been applied with impressive results in mammalian and amphibian systems. Electroporation can be used in situ to transfect neurons and glia in the central nervous system (CNS) of the tadpole (Fig. 1). For single-cell electroporation, the tip of a DNA-filled glass micropipette is placed against the cell to be electroporated (under visual guidance provided via an upright microscope), and current is passed through the pipette. This transiently disrupts the plasma membrane and ejects the DNA (Haas et al. 2001; Bestman et al. 2006). For transfecting larger numbers of cells, DNA solution can be pressure-injected into the brain ventricle or directly into the tissue, and current passed through the target tissue using a pair of closely spaced platinum plate electrodes (Haas et al. 2002; Falk et al. 2007). Both methods are effective and versatile for labeling muscle cells and cells in the CNS (e.g., neurons, glia, and proliferating cells) in *Xenopus* tadpoles. In contrast to viral or transgenic methods, DNA plasmid expression vectors purified from bacteria, even by a simple miniprep, can be used to electroporate neurons directly. However, cleaner DNA preparations—we typically use endotoxin-free maxipreps—typically result in healthier cells and higher transfection efficiencies. Moreover, multiple constructs can be used to cotransfect the same cells with high efficiency, by simply electroporating the cells with a cocktail of different plasmids mixed together.

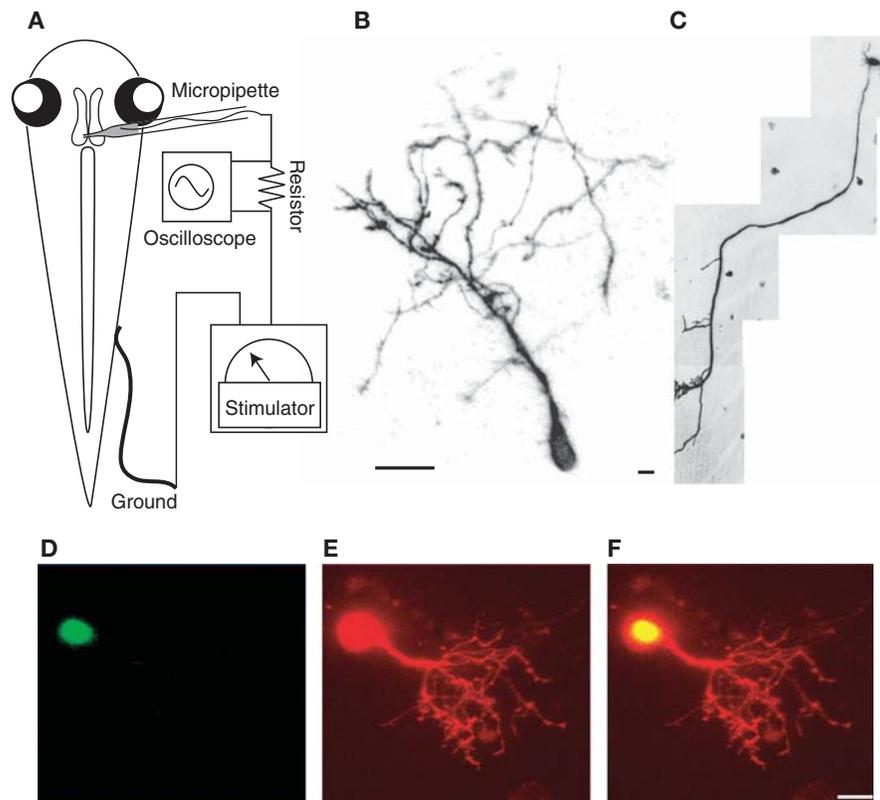


FIGURE 1. Single-cell electroporation. (A) Circuit diagram and electrode placement schematic for tracer or DNA electroporation of single tectal neurons in tadpoles. (B) Tadpole tectal neuron transfected by electroporation with plasmid encoding farnesylated EGFP. (C) Example of electroporated motor neuron in spinal cord. (D–F) Coelectroporation of fluorescently tagged Morpholino oligos (D) with Texas red dextran (E). (F) Merged image. (D,E, Modified, with permission, from Haas et al. 2002.) Scale bars, 20 μm .

In principle, any two molecules that migrate together in an electric field can be used to cotransform cells by electroporation. For example, it is possible to electroporate fluorescent dextrans or plasmid DNA to reveal the morphology of a single cell, together with fluorescently tagged antisense morpholino oligonucleotides to inhibit the expression of specific gene products in that cell (Haas et al. 2002; Bestman et al. 2006). Expression levels under the control of the cytomegalovirus promoter are extremely high, with GFP typically completely filling tectal dendrites between 12 and 24 h and reaching the distal tips of retinal ganglion cell axons by ~48 h following electroporation. Use of constructs driven by different promoters, such as the weaker neuron-specific enolase promoter or the GABAergic neuron-specific VGAT promoter, provides added versatility to this method. Amplification of expression from weak cell-type-specific promoters can be accomplished using the gal4/UAS system, as has been used extensively in *Drosophila* and zebrafish.

There are a variety of fluorescence-based methods for visualizing individual neurons in living *Xenopus* tadpoles. These include two general approaches to single-cell visualization: (1) targeted introduction of fluorophore in the form of dye or genetic material into just one cell at a time (see **Labeling Individual Neurons in the Brains of Live *Xenopus* Tadpoles by Electroporation of Dyes or DNA** [Ruthazer et al. 2013a]) and (2) bulk transfection of multiple cells in a manner that permits single cells to be visualized independently by activation of fluorophore (see **Dye Labeling Retinal Ganglion Cell Axons in Live *Xenopus* Tadpoles** [Ruthazer et al. 2013b]; **Bulk Electroporation of Retinal Ganglion Cells in Live *Xenopus* Tadpoles** [Ruthazer et al. 2013c]). The first approach has the advantage of producing true single-cell chimeric animals in which to study the effects of overexpression or knockdown of a gene in an otherwise entirely wild-type background. The second approach becomes useful under conditions in which it might be too difficult to reliably perform juxtacellular electroporation because of limited visibility or access.

ADDITIONAL CONSIDERATIONS

Imaging Labeled Neurons in the Living Tadpole Brain

For repeated imaging at intervals of hours or days, animals can be chronically mounted in an imaging chamber. Animals are sedated by immersion in a dilute anesthetic solution and mounted under a coverslip in a silicone polymer (Sylgard) chamber custom-shaped to hold the animal in the same orientation over repeated imaging sessions. The animal should sit snugly in the chamber with its dorsal aspect immediately under the coverslip. The animal should not be compressed by the coverslip or by the walls of the chamber, but the chamber should seal tightly against the coverslip to prevent movement during imaging, especially if immersion objectives are to be used. Between images the tadpole can be removed from the chamber and returned to normal rearing solution to recover. If animals of different stages are to be imaged or if images are to be collected over a period of days during which the animals grow, separate chambers must be made for the different-sized animals. A good chamber will cause the animal to settle into roughly the same position and orientation each time it is imaged, facilitating later alignment of images.

For short interval imaging (i.e., on the order of second or minute intervals), it often is best to immobilize animals by embedding in low-melt agarose (e.g., 1% in rearing solution). The agarose-embedded tadpole can then be placed in a perfusion chamber on the microscope stage. For ultrastable conditions, the embedded tadpole can be further enclosed in a rearing solution-filled chamber under a cover glass. When it is necessary to omit anesthetics, for example, when studying activity-dependent processes, subcutaneous injection or bath application of a paralytic agent such as 1 mM tubocurarine can be used to immobilize the animal.

Image Analysis and Morphometry

One of the most critical and time-consuming steps in three-dimensional (3D) time-lapse imaging is data analysis. There are three fundamental morphological properties of neurons that can be extracted

from images: total arbor size (linear length or coverage volume), individual branch tip number and length, and arbor complexity (e.g., branch density, branch order). Time-lapse imaging adds further useful information about growth rates and branch tip dynamics that would be impossible to extract from static images in fixed material. To take full advantage of these data, it is important to use a strategy for morphometric analysis that incorporates both structural and temporal information.

The ideal software should perform automatic tracing of dendritic and axonal arbors and be able to identify the same branch tip at each time point. Because some errors are inevitable, a user-friendly editor should allow drawings of branches to be compared with the raw data and modified easily. Automated neuron reconstruction can be much faster, but far less accurate than a manual reconstruction performed by an experienced researcher. Although a few professional reconstruction software packages do offer adequate automated drawing, these are generally quite expensive; several useful freeware programs have also been released in recent years.

We perform manual reconstructions using Object-Image (Vischer et al. 1994), a freely distributed version of the popular and powerful NIH Image analysis software for the Macintosh. Unfortunately, this software is no longer supported in the latest version of the Macintosh operating system, but it can still be run on any older system that includes the Classic OS9 emulator. This program permits a nondestructive overlay to be traced manually in three dimensions on a confocal z-series stack (see Fig. 2). By simultaneously running a set of custom macros for time-lapse analysis, it is possible to assign an identity number to each unique branch tip and thus follow it through the time series (Ruthazer and Cline 2002).

Ultimately, for any analysis program to be useful for time-lapse analysis, it must be able to generate a list of branch tips, their 3D coordinates, and their lengths at each time point. This information can then be analyzed easily to investigate aspects such as branch growth rates, branch tip dynamics, and lifetimes. We also have created Object-Image macros to perform more elaborate analyses, such

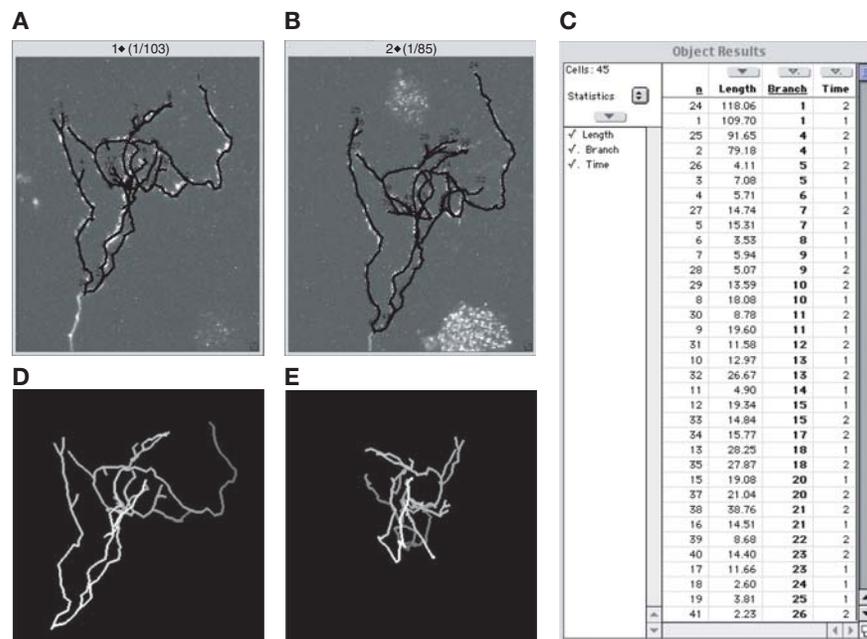


FIGURE 2. Time-lapse axon reconstruction using Object-Image software. (A) Projection of z-series captured at first time point. The manually traced overlay (black lines and numbers) shows the object identifier number at the tip of each branch during tracing. (B) Projection of z-series from second time point. Normally, tracing would be performed in three dimensions on the stack rather than on a projected image. Tracing in three dimensions permits true branch growth to be distinguished from apparent changes caused by shifts in the orientation of the animal. (C) Object Results list generated during tracing that identifies each individual branch with a branch number and time information permitting its growth and dynamics to be followed over time. (D) Rotator ray trace image of axon from A, which can be viewed from all angles. (E) Same axon rotated perpendicularly as it would appear looking down the main axon shaft.

as 3D Sholl analyses on the reconstructed arbors (Ruthazer and Cline 2002). All of these macros are available, with detailed instructions, from <http://www.scripps.edu/cb/cline/cgi-bin/main.pl?p=Methods>.

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