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Optical Projection Tomography as a Novel Method to Visualize and Quantitate Whole-Brain Patterns of Cell Proliferation in the Adult Zebrafish Brain

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Abstract

How distinct cell populations are distributed in three-dimensional space under homeostasis or following injury, neurodegeneration, or with senescence can teach us much about brain-wide patterns and signaling along the neuroaxis. Visualizing individual cell populations in the mature vertebrate central nervous system (CNS) has remained a challenge as a result of difficulty clearing adult brain tissue or limitations in imaging depth or resolution. We have developed a simple clearing and imaging pipeline optimally suited for the adult zebrafish brain to investigate changes in patterns of cell proliferation in wild-type and transgenic backgrounds that can easily be quantified and represented using FIJI and IMARIS software.

Keywords: three-dimensional imaging, clearing, neurogenesis, regeneration, brain development, stem cell proliferation

Methods

The zebrafish has become a popular model in neuroscience to study changes arising in the mature central nervous system (CNS) as a result of social behaviors, neurogenesis, brain injury and disorders, regeneration, neuroimmune interactions, and neurodegenerative diseases.[1–10] Unfortunately, few brain-wide imaging tools are available to researchers investigating changes in the CNS during adulthood. Most studies continue to be performed by the standard method of fixing whole adult brains, sectioning, and confocal imaging antibody markers of interest. However, serial sectioning is time-consuming and severely limits interpretations of changes occurring across the entire brain axis. By contrast, the ability to visualize markers in three-dimensional (3D) space allows investigators to screen the adult brain for regions of interest following manipulation. In this study, we have developed an efficient pipeline to clear, visualize, and quantitate the whole adult zebrafish brain using optical projection tomography (OPT) to assess changes in cell proliferation alone or in combination with transgenic reporter lines.

The small size of the adult zebrafish brain ($\sim 6 \times 3 \times 3$ mm; Fig. 1A) makes it ideally suited for OPT imaging as a tool to gain new insight into brain-wide cellular patterns in vertebrates. OPT allows imaging of fixed specimens at near cellular resolution (3.21 μm/pixel) with three or more fluorescent channels from UV to the IR spectrum (typically excitation at 350, 488, 555, and 647; Fig. 1B–D, K, L), as well as brightfield, which can be reconstructed to obtain a 3D view of organs. This method was originally used to study mouse embryo development,[11] but has more recently

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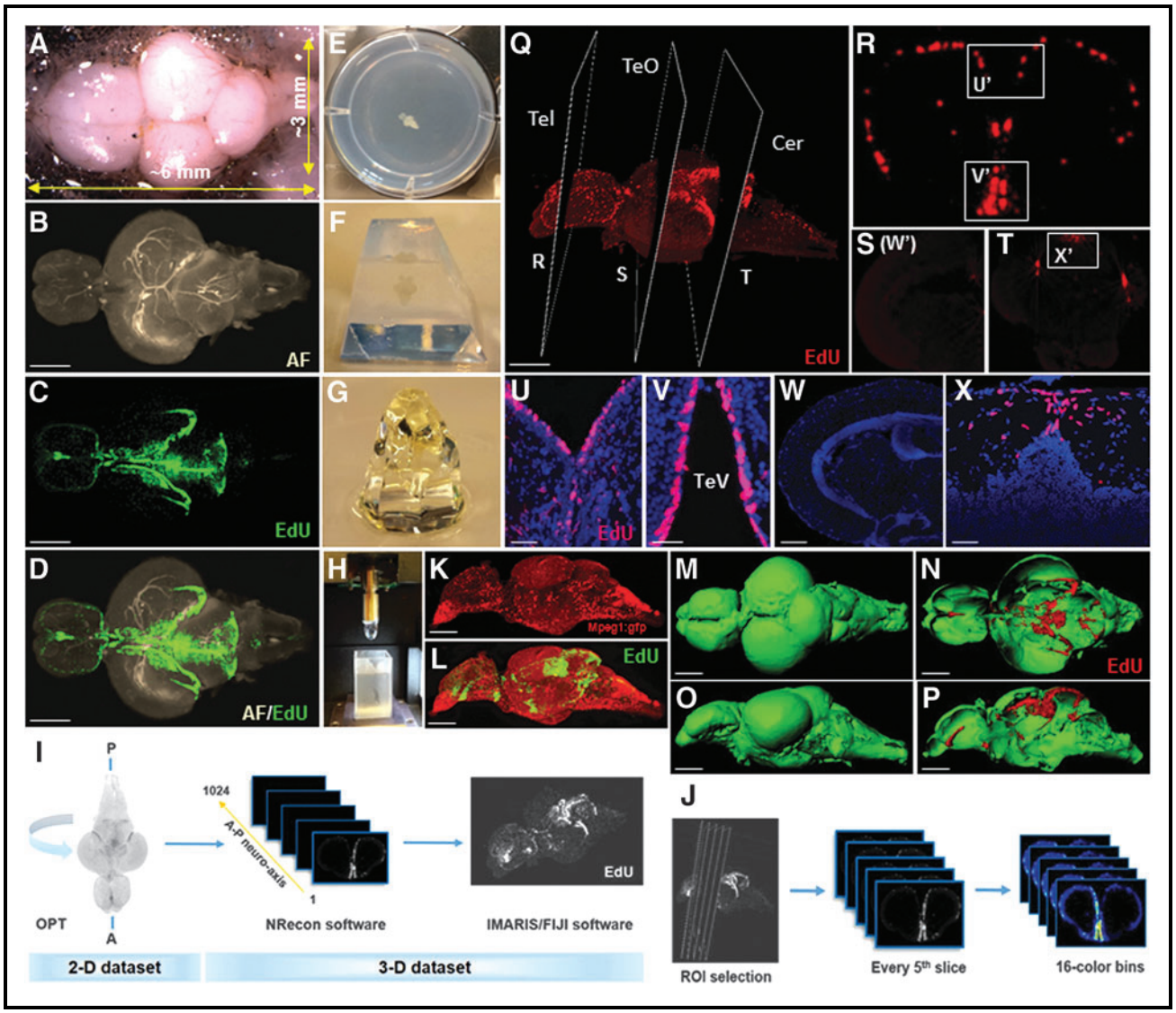

FIG. 1. Sample preparation, output, and quantification of cleared adult zebrafish brains imaged using OPT. **(A)** Top view of adult zebrafish brain with approximate dimensions of length and width in 6-month to 1-year-old animals. **(B–D)** Reconstructed OPT images showing autofluorescence (AF) scanning of brain volume in the 488 channel (*pale yellow*; **B**), EdU scanning of adult proliferative zones (*green*; **C**), and merge **(D)**. **(E–H)** Brain embedded in low melting agarose **(E)**, trimmed and cleared **(F, G)**, and mounted for OPT imaging **(H). (I)** Representation of specimen positioned along the long axis for OPT imaging, reconstructed OPT dataset in 1-pixel depth sections ($\sim$1024 sections) using NRecon software, and 3D view of EdU labeling in adult brain using IMARIS. **(J)** Example of reconstruction and quantification of OPT dataset using FIJI software to examine adult proliferation zones in the forebrain by selecting the ROI, and analyzing every fifth section using 16-bit color to bin EdU intensity levels. **(K, L)** Midsaggital view of a reconstructed multichannel OPT scan of EdU and GFP in the Tg(mpeg1:gfp) macrophage-specific transgenic line. **(M–P)** Dorsal **(M, N)** and lateral **(O, P)** views of isosurface-rendered adult brains (*green*) using IMARIS software with EdU labeling (*red*). Isosurface-rendered brains were derived from initial autofluorescence scans, such as that shown in **(B)**. **(Q–T)** 3D-reconstructed OPT dataset depicting reliability of EdU penetration and staining in the forebrain **(R)**, optic tectum **(S)**, and cerebellum **(T)** compared with conventional cryosectioning with EdU staining in a similar domain of a separate specimen **(U′–X′** denote regions shown in **U–X)**. **(U–X)** EdU labeling (*pink*) in 18-μm thin cryosections of the adult zebrafish brain in the dorsal **(U)** and ventral **(V)** forebrain, optic tectum **(W)**, and cerebellum **(X)**. Blue counterstain; 4,6-Diamidino-2-phenylindole. Scale bars: **(B–D, K–Q)** 500 μm; **(U–X)** 10 μm. 3D, three-dimensional; EdU, 5-ethynyl-2′-deoxyuridine; OPT, optical projection tomography; ROI, region of interest.

been applied to organ morphogenesis[12] and the distribution of neural stem cell niches.[13] Compared with more recent imaging technologies such as light sheet microscopy, OPT offers isotropic scanning of specimens that can be readily cleared in a simple mixture of benzyl benzoate: benzyl alcohol. In addition, reconstruction is simple and fast and datasets are compatible with FIJI or IMARIS for quantification and visualization. Below we present an inexpensive and straightforward method to examine patterns of cell proliferation in the adult brain or other adult organs, which can be performed in wild-type, transgenic, or mutant backgrounds.

Brief overview of workflow:

1. Labeling proliferating cells: Inject adult zebrafish twice intraperitoneally with 40 μL of a 10 mM solution of the *S*-phase marker 5-ethynyl-2′-deoxyuridine (EdU) over 4-h to label proliferating cells in the CNS.
2. Tissue processing: Sacrifice animals and dissect out the brain. Fix samples in 2% paraformaldehyde overnight. Rinse samples in phosphate-buffered saline (PBS, pH 7.4) with 0.3% Triton X-100 for 2-h, followed by 1 × -PBS with 1% Triton X-100/5% dimethyl sulfoxide for 24-h.
3. Staining proliferating cells: Commence EdU staining over 4 days in 12-well plates using EdU staining solution with Alexa Fluor Azide dyes (Supplementary Table S1; Supplementary Data available online at www.liebertpub.com/zeb). In addition, immunohistochemistry can be performed after EdU staining.
4. Embedding: Embed samples in a 1% solution of distilled water and low melting agarose in a six-well plate. Position samples in the centre of well with the brain oriented dorsally. Once agarose is set, trim brains (Fig. 1E–G).
5. Dehydration and clearing: Dehydrate samples in 100% methanol, providing three to four solution changes over 24 h. Clear samples in a 2:1 solution of benzyl benzoate: benzyl alcohol as above, over 24 h (Fig. 1G).
6. OPT scanning and reconstruction: Adhere cleared brain samples onto OPT mounts (Fig. 1H). Image brains using desired parameters on a Bioptonics 3001 OPT scanner (Bioptonics, Edinburgh, United Kingdom; Supplementary Table S2) and reconstruct using Nrecon software (Bruker microCT; Fig. 1I).
7. Quantification and visualization: Import final reconstructed datasets into FIJI or IMARIS software to analyze fluorescence by intensity, surface area, or volume (Fig. 1J), and visualize single or multichannel data as 3D maximum projections (Fig. 1K, L) or isosurface-rendered images (Fig. 1M–P).

Work in our laboratory using this imaging approach to examine adult neurogenic compartments in the zebrafish brain has shown this method to be highly consistent in EdU staining compared with our standard cryosectioning protocols, demonstrating reliable penetration of EdU through the entire adult brain (Fig. 1Q–X, Supplementary Movie SM1). The protocol is compatible with fluorescent proteins and green fluorescent protein (GFP) reporter lines, for example, microglia can be visualized in the brain using the Tg(mpeg1:gfp) line[14] (Fig. 1K, L).

We envision this method to be an excellent tool to investigate brain-wide changes in proliferation postlesion (Supplementary Fig. S1), brain structure development and senescence, or to screen for proliferative abnormalities in mutants. Unlike many of the more advanced clearing and imaging methods today, no specialized reagents or equipment are required for this method, making it accessible to most laboratories. We welcome collaborations for those interested in taking advantage of this method and provide a more detailed protocol in the Supplementary Data.

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Disclosure Statement

No competing financial interests exist.

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