In vivo long-term continuous observation of gene expression in zebrafish embryo nerve systems by using harmonic generation microscopy and morphant technology

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1 Introduction

Genes are the chemical, physical, and functional units of heredity. Genes, which are made up of DNA, act as instructors in the production of proteins. Gene expression plays an important role in embryo development and organ function. The vertebrate genome contains about 50,000–100,000 genes, many of which have unknown functions. In this study, we focused on the continuous long-term in vivo observation of gene expression in the nervous system. Because the nervous system has a significant influence on sensory reactions, body actions, and organ function, we decided to investigate the genes involved in nerve development.

Many live imaging systems are used to study gene expression in neurology. Magnetic resonance imaging (MRI) determines which parts of the brain are activated by different types of physical sensation or activity, such as the movement of a subject’s fingers, visual stimulus, and auditory stimulus. MRI is also used to detect the overexpression of genes in the brain, but this technique needs sensitive magnetic resonance contrast agents. Ultrasound can be used for determining the developmental effects of targeted genetic or cellular interventions at critical stages of placentation, gastrulation, axis formation, and neural tube closure. However, this technique...
cannot reveal neural activity down to the cellular level. Confocal fluorescence and multiphoton fluorescence microscopy can study some in vivo changes in gene expression during embryo development, but these techniques are limited by the binding and expression time of fluorescence proteins. Harmonic generation microscopy (HGM), which is known to leave no energy deposition on the interacted matter due to HGM’s energy conservation characteristic, supports the least optically invasive nature desirable for in vivo long-term continuous observation in studying developmental processes. Similar to confocal and multiphoton microscopy, HGM is used with subcellular-level 3-D resolution, while the technique’s millimeter penetration capability makes HGM an excellent tool for studying gene expression during embryogenesis. Without the aid of fluorescence protein, nerve fibers and spindle fibers can be directly revealed through the second-harmonic generation (SHG) signals, and cell membranes can be directly revealed through the third-harmonic generation (THG) signals, making HGM an ideal tool for studying nerve fiber formation, cell division activity, and organ morphology related to different gene functions.

The morphant technique is straightforward to perform, competent for action in all cell types, efficient at depleting the selected protein, and amenable to the targeting of many genes. For gene function studies, it can act as an in vivo gene targeting tool. Morpholino phosphorodiamidate oligonucleotides [morpholinos (MOs)] are synthetic DNAs that contain a MD ring and a neutral charge backbone and have high affinity to ribonucleic acids (RNAs). By binding to and blocking the translation of mRNAs, MOs have been successfully used in a variety of systems for gene knockdown studies. The zebrafish, being a standardized vertebrate model, is viewed as an appropriate animal model for morphant technology. Zebrafishes have definite developing stages, their genome sequence has been completely discovered, and much of their genetic material is the same as that of humans. The zebrafish’s rapid rate of development, precisely defined developing stages, transparency, small size, and external development capability facilitate many genomic studies and microscopic observations. Formal optical techniques used in morphant technology research, such as staining, biopsy, and optical microscopy, cannot in vivo trace the gene expression in the same living zebrafish for an extended period. Optical higher-HGM can continuously observe zebrafish embryos, including cell morphology of the nervous system, for over 20 h with no photodamage effects, making it ideal for morphant technology research.

In this paper, we report the application of HGM for continuous observations during in vivo morphant zebrafish embryo studies. We focused our studies on the genes zarn2α (aryl hydrocarbon receptor nuclear translocator) and hif (hypoxia inducible factor)-1α, 2α, 3α because of their influence on the nervous system from the very beginning of embryo development. Weak cell adhesion during the gastrula period and cell apoptosis during the pharyngula period were revealed through THG modality. With SHG modality, the decrease in nerve fiber formation in the forebrain of the embryos due to the blocking of these genes could be observed. Our study indicates that combining in vivo continuous HGM imaging with morphant technologies can offer valuable insight into gene functions in vertebrate embryo development, especially in the early developing stage, complementary to other existing technologies.

2 Materials and Methods

Our laser-scanning harmonic generation microscope was adapted with an Olympus FV300 scanning unit along with an Olympus BX51 microscope. The collimated Cr:forsterite laser beam, whose center wavelength was 1230 nm with a repetition rate of 110 MHz and a pulse width of 140 fs, was coupled into the scanning system as the excitation source. To observe the whole 3-D structure of the zebrafish brain, we used a 2-mm-working-distance, high-numerical-aperture (NA) infrared objective (LUMPPlanFI/IR 60X/water/NA0.9, Olympus) to focus the laser beam on the desired location of the specimen with a spot size close to its diffraction limit. The average power after the objective was approximately 150 mW. Taking advantage of the transparency and finite thickness of the live specimen, we used a NA0.9 air condenser to collect higher harmonic generation signals in the forward direction. The SHG and THG signals were separated by a beam splitter and were guided into two photomultiplier tubes (PMTs) to record the filtered SHG and THG signals, respectively. For the THG signal origin study, filtered three-photon fluorescence with a center wavelength at 535 nm was collected using the focusing infrared objective in the epi-direction for PMT detection. The zarn2α-specific morpholino-modified-antisense-oligonucleotides (MOs) were designed to target the zebrafish ARNT2A (GenBank AF055166) (5′-GTTTACACGCGCTGTTGTTGC-3′) cDNA (nt 221–245). The hif-1α-specific MOs were designed to target the zebrafish hif-1α (GenBank AY326951) (5′-CAGTGAACACTCCAGTATCCATTCC-3′) cDNA (nt 235–259). The hif-2α-specific MOs were designed to target the zebrafish HIF-2α (GenBank DQ375242) (5′-CGCTGTGCTCGGTAATCCGGCCAG-3′) cDNA (nt 223–247). The hif-3α-specific MOs were designed to target the zebrafish HIF-3α (GenBank AY330295) (5′-CCTTTTCGACGTAGGTTACCACATC-3′) cDNA (nt 92–116). The four MOs were obtained from a commercial supplier (Gene Tool, Philomath, OR) and were microinjected into the zebrafish embryos. The dosage of MOs represents the total weight of MOs injected into one embryo. For example, a dose of 12-ng zarn2α MOs were injected, but a mixed dose of 18-ng hif-1α, 2α, 3α means that 6-ng hif-1α MOs, 6-ng hif-2α MOs, and 6-ng hif-3α MOs were injected simultaneously into one embryo. These genes have great influence on the nervous system, including the forebrain, midbrain, and somite. In a preliminary study we observed that in separate hif-1α, hif-2α, or hif-3α morphant zebrafish embryos, the blocking of the hypoxia response was not full, probably because hif-1α, hif-2α, and hif-3α MOs have similar functions, so they have a genetic redundancy problem. To overcome this problem, we completely blocked the hypoxia response by injecting hif-1α, hif-2α, and hif-3α MOs simultaneously. Because of the ease of identification and observation, we focused on the development of the forebrain. For each embryo, we performed continuous HGM observation for over 17 h, and observation of different morphant zebrafish embryos was...
repeated more than 10 times with different embryos. Although there were variations between morphants of a single type, the tendencies of decreasing nerve fiber and neural tube distortion in the forebrain, as discussed below, were much the same in all embryos. Therefore, only one of the typical results representing different morphant zebrafish is shown in the following discussion. Whole-mount in situ hybridization was also performed as previously described. To make gene-specific probes, digoxigenin-labeled cRNA fragments containing 1069 bp (nt 1800–2850) of the R-cadherin gene (GenBank BC116496) and 500 bp (nt 55–554) of the HuC gene (GenBank U62018) were synthesized with the DIG RNA Labeling Kit (Sp6/T7, Roche, USA). Hybridization was detected by anti-digoxigenin (DIG) antibody coupled to alkaline phosphatase. For controls, a random sequence morpholino (the standard control morpholino) available from Gene Tools was used as an injection control. For the zarnt2a morphant zebrafish embryo experiments, a dose of 12-ng random sequence MO was injected into the embryos as a control, while in the hif-1a, 2a, 3a morphant zebrafish embryo experiments, a dose of 18-ng random sequence MO was injected into the embryos as controls.

3 Results and Discussions

Because of the Gouy phase shift effect in a tightly focused beam, THG occurs when the focus is located at the interface of two media with different linear or nonlinear optical indices. Because SHG can arise from highly organized nanostructures with an ordered arrangement of asymmetric biomolecules where the optical centrosymmetry is broken, SHG can reveal spindles and nerve fibers. Northern hybridization analysis study and in situ hybridization showed that zARNT2A mRNA was transcribed in the brain and has great influence on the nervous system. However, it is not known how zARNT2A mRNA influences nerve development. Therefore, the zarnt2a morphant zebrafish serves as a suitable animal model to test if continuous HGM observation can be combined with morphant technology for gene expression studies. At the very beginning of zebrafish embryo development, we observed a conspicuous diversity between the control and the zarnt2a morphant zebrafish embryos. Figure 1 shows the THG-sectioned in vivo microscopic images from the two types of embryos at 7-h postfertilization (hpf). Epiboly, which begins in the late blastula period, causes the thin-
ning and extending of both the yolk syncytial layer (YSL) and the blastodisc over the yolk cells.\textsuperscript{18} The yellow arrow in Fig. 1(b) indicates the position of the ectoderm, and the red arrow indicates the position of the mesoderm formed by the migrating cells. Previous THG studies,\textsuperscript{7,8,10,12,19} have already indicated that THG modality can reveal cell membranes. As clearly shown in the THG microscopic images of the zarnt2a morphant zebrafish embryos, some cells can be found to be isolated [white arrow in Fig. 1(c)] with a round shape during epiboly through THG modality. This is an indication that cell adhesion becomes weak due to the blocking of the zR-cadherin (zcdh-4) expression of in cell–cell adhesion.

R-cadherin and N-cadherin are both Ca\textsuperscript{2+}-dependent cell–cell adhesion molecules. Moreover, they can form a cis-heterodimer to generate new functional units to mediate cell–cell adhesion.\textsuperscript{26,27} To further investigate the HGM-observed weak cell adhesion in zarnt2a morphants, we analyzed the expression of zR-cadherin (zcdh-4) of the control and zarnt2a morphant zebrafish embryos at 7 hpf. As shown in Fig. 2, at 7 hpf we observed that zR-cadherin (zcdh-4) was expressed widely [Figs. 2(a) and 2(e)] in the control zebrafish embryos but was expressed much less in zarnt2a morphant zebrafish embryos [Figs. 2(b) and 2(f)]. Even at the 72-hpf stage, the expression of zR-cadherin (zcdh-4) in the zarnt2a morphant [Figs. 2(d) and 2(h)] was still significantly less than the expression in the control embryos [Figs. 2(c) and 2(g)]. Our analysis further confirms our frequent HGM observation that the knockdown of the zarnt2a gene in zebrafish causes defects in cell–cell adhesion.

As the long-term in vivo observation continued, the same embryo developed to the 12-hpf stage (shown in Fig. 3). Compared with the early-grown straight nerve fibers (revealed through SHG modality) in the neural keel of the control set, the SHG signals in the neural keel of the zarnt2a morphant zebrafish embryos were found to be much fewer and weaker. Our continuous observation revealed that in the control set, in the zebrafish embryos’ forebrain, the lumen formed through cavitation, and the SHG signals revealed that numerous nerve fibers had developed in the neural tube at approximately 24 hpf.\textsuperscript{12} However, due to the lack of zARNT2A proteins, the SHG signals in the forebrain of the morphant zebrafish revealed the greatly reduced nerve fiber development compared to the controls (Fig. 4). Greatly suppressed nerve fiber development in the zarnt2a morphant zebrafish’s forebrain, indicated by the abatement and weakening of the SHG signals, might be directly or indirectly related to the observed weak cell adhesion revealed by the THG signals during the gastrula period. According to the fate map of zebrafish, the fate of the blastoderm cells appears to be fixed shortly before gastrulation begins.\textsuperscript{18} The cells in the ectoderm will give rise to such tissues as the epidermis, the central nervous system, and the neural crest. The weak cell adhesion during gastrulation caused by the lack of zARNT2A would influence the formation of the three germ layers and could thus lead to the later reduction in nerve fiber development in the forebrain, as indicated by the SHG signals.

Because continuous HGM observation of the zarnt2a morphant zebrafish had successfully identified some zarnt2a gene function in the nervous system from the very beginning of embryogenesis, we applied HGM to study the influence of the...
unknown hif-1α, 2α, 3α genes, which have partial overlap with the zarrt2a gene in sequence, on nerve fiber development. Figure 5 shows an example of in vivo HGM-sectioned images of the forebrain of the control zebrafish and the hif-1α, 2α, 3α morphant zebrafish with different applied dosages at 24 hpf. Compared with the control zebrafish [Fig. 5(b)], the development of nerve fibers was found to be considerably diminished, while serious distortion of neural fibers could be observed when the MO dosage increased. This MO dosage-dependent study suggested that hif-1α, 2α, 3α is related to the formation of nerve fibers in the brain.

To identify the function of the hif-1α, 2α, 3α genes in development of the nervous system more specifically, we quantitatively analyzed the SHG signals of the nerve fibers in the forebrain of the hif-1α, 2α, 3α morphant zebrafish. The ventricular zone is approximately 10 μm wide from the edge of the neural tube.22 In this region, the SHG signals arise from both the spindle fibers of the neural stem cells and the nerve fibers. Therefore, we selected six zones with the same area size in the neural tube outside the forebrain ventricle zone (more than 10 μm from the edge of the neural tube) for each dose (Fig. 5). Thus, we made sure that the SHG signals we analyzed all arose from the nerve fibers. With the same PMT voltage of 1100 V, images of 512 × 512 pixels, integration time of 1.3 s, and laser power of 125 mW after the objective lens, the SHG signal pixels with contrast lower than 20 were considered to be noise. Figure 6 shows the average SHG signal pixels of six zones with contrast above 20. The figure shows that the SHG signals decrease as the MO dosage increases, and there exists an obvious gap between the control zebrafish embryos (indicated as dosage 0 ng) and the morphant zebrafish embryos, proving that hif-1α, 2α, 3α is essential for nerve fiber formation.

According to previous studies,22 the gene zHuC was expressed in the neuronal precursor cells in the neural plate immediately after gastrulation and then expressed strongly in most regions of the nervous system.22 Therefore, zHuC, one of the earliest neuronal markers, is an excellent marker gene for observing the developing nervous system in zebrafish embryos. In order to confirm the above result, we examined zHuC expression in the control zebrafish embryos and the hif-1α, 2α, 3α morphant zebrafish embryos of different doses (9 ng, 18 ng, 27 ng) at 24 hpf. Compared with the control zebrafish embryos, the hif-1α, 2α, 3α morphant zebrafish embryos all revealed minor expression of zHuC in the forebrain (Fig. 7), which is consistent with the previous result. Hence, these studies suggest that the number of neural cells in the forebrain decreased when the MO dosage increased [Figs. 7(b)–7(d) and 7(f)–7(h)].

In the HGM images we can observe not only the decreasing number of nerve fibers caused by knocking down the hif-1α, 2α, 3α genes, but we can also find widespread apoptosis cells in the forebrains. In the in vivo-sectioned HGM images in the developing forebrain of the hif-1α, 2α, 3α morphants, one can observe many round, solid THG objects.
Fig. 7 Expression of zHuC is less and less with the dosage of MO increased. Lateral (a), (b), (c), (d), dorsal (e), (f), (g), (h) views of embryos at 24 hpf. The expression of zHuC in the forebrain of control zebrafish (a), (e) is very strong, but the zHuC expression in hif-1α,2α,3α [9 ng: (b), (f)], [18 ng: (c), (g)], and [27 ng: (d), (h)] morphant zebrafish embryos is much less when the dosage of MO increased. F, forebrain; M, midbrain; H, hindbrain.

Fig. 8 In vivo (b) THG (shown in purple) and the simultaneously acquired (c) 3PF (shown in yellow) images of the acridine orange stained hif-1α,2α,3α morphant zebrafish embryo forebrain at 20 hpf. (d) The combined THG/3PF image shows that the round-shaped THG signals were from the apoptotic cells. The yellow arrow shows the posterior-to-anterior direction. NT, neural tube. The black line in (a) shows the sectioned region. Scale bar: 50 μm.

Fig. 9 (a) Nonlinear emission spectrum from the acridine orange labeled apoptotic cells showing the THG peak at 410 nm and the broad 3PF with a center wavelength of 535 nm. (b) Power dependency of the THG signals (triangle) and the 3PF signals (rectangle) of the acridine orange labeled apoptotic cells. The well-matched solid lines are the slope=3 fitting, confirming the three-photon nature of both nonlinear processes.

(red arrows in Fig. 5) which were sometimes so dense that they appeared as connected regions. [Please compare the neural tube areas in Fig. 5(b) to Fig. 5(c)-(e).] To study the source of these round objects reflected through the THG contrast in the neural tube, we performed many simultaneous staining experiments, and our analysis indicated that the contrast came from apoptosis cells. Figure 8 shows the simultaneously acquired in vivo THG and three-photon-fluorescence (3PF) image of the hif-1α,2α,3α morphant zebrafish embryo with acridine orange staining (A6014, Sigma-Aldrich, emission central wavelength 535 nm).21 The 3PF signals from the acridine orange stain labeled the apoptotic cells. The nonlinear emission spectrum of the acridine orange-stained apoptotic cells is given in Fig. 9(a), showing the narrow-linewidth THG at 410 nm and the broad 3PF between 525 and 575 nm. The cubic power dependencies of both the 3PF intensity from the acridine orange (centered at 535 nm) and the THG intensity (centered at 410 nm) are shown in Fig. 9(b), confirming the three-photon nature of the acridine orange fluorescence. Our 3PF study indicated that the observed solid THG objects are apoptotic cells.

Our study not only presented evidence that apoptotic cells can provide strong THG contrast but also demonstrated strong apoptosis behaviors in the developing forebrain of the studied hif-1α,2α,3α morphants. More detailed, long-term in vivo investigations [Fig. 10(b)] revealed abundant mitosis behaviors near the midline of the neural rod through the SHG signals from the mitosis spindles in hif-1α,2α,3α morphant zebrafish embryos. Combined with the previous results of strongly decreased nerve fibers, our study indicates that in hif-1α,2α,3α morphant zebrafish, the lack of HIF-1α,2α,3α proteins does not influence the mitosis of the neural stem cells, which are located in the ventricular zone near the midline of the neural rod,12 but influences the formation of nerve fibers. This could be the first in vivo multimode microscopy that includes SHG, THG, and 3PF signals simultaneously.

4 Summary
With a deep penetration depth and minimal attenuation in living tissues, a 1230-nm Cr:forsterite laser is ideal for in vivo long-term continuous biological study. Combining the least invasive Cr:forsterite laser-based HGM with well-developed
morphant technology could reveal the role of different genes without staining. In our in vivo study of the zarnt2a and hif-1α,2α,3α genes in nervous system development, by blocking the expression of these genes, the THG signals revealed weak cell adhesion from the very beginning of embryogenesis, thus affecting the formation of germ layers, which determine the destination of all cells in the embryos. The distortion of the neural tube in the forebrain observed by the THG signals and the decrease in nerve fiber formation observed by the SHG signals indicated that these genes play important roles in the development of the nervous system. In addition to morphology observation, quantitative analysis of the SHG signals in the neural tubes demonstrated the influence of MOs on nerve fiber development through dosage-dependent studies. The increased MO dosage led to a quantitative decrease in the number of observed SHG signals in the nerve fibers, and the SHG signal intensity apparently varied between the control and the morphant zebrafish embryos. This proves that blocking the hif-1α,2α,3α genes influences the formation of nerve fibers. By using in vivo staining, we have also successfully proven that THG can reveal apoptotic cells. Through long-term continuous observation, our HGM study also suggested that instead of preventing neural stem cells from undergoing mitosis, the lack of hif-1α,2α,3α genes affected the formation of nerve fibers. This study shows that combining morphant technology with continuous in vivo HGM observation can provide a new tool for studying gene function in embryology, not only in neurology but from the very beginning of embryogenesis.

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