Noninvasive Multi-Modality Nonlinear Imaging of Heart Development Using Transgenic Zebrafish Lines Tagged with Hc-Red Fluorescence Proteins

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Abstract: We generated zebrafish lines with two-photon red fluorescence expressed in the heart excited by 1230nm light. With high penetration and high viability, heart development dynamics deep inside live embryos is noninvasively revealed in vivo.
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Transgenic lines carrying a specific tissue tagged by green-fluorescence-protein (GFP) live markers have been a powerful tool for the developmental biology study because they encapsulate the expression of endogenous genes [1]. Traditionally with a two-photon fluorescence microscope (2PFM) based on a femtosecond Ti:sapphire laser (with a wavelength between 700-980nm), green fluorescence can be excited by simultaneous absorption of two photons for high-resolution three-dimensional (3D) optical imaging [2]. However for future *in vivo* biological studies, Ti:sapphire-laser based optical technology still presents several limitations including finite penetration depth, strong on-focus cell damage, and multi-photon phototoxicity. For high optical penetration and minimized photodamages, two-photon molecular imaging based on light sources with an optical wavelength located around the biological penetration window (~1300nm) is desired, where unwanted light-tissue interactions including scattering, absorption, and multi-photon photodamages can all be minimized.

Previous experiments around the optical penetration window indicated inefficient green fluorescence excitation of GFP through three-photon absorption. Red fluorescence proteins with an emission wavelength close to near-IR is thus highly desired for future non-invasive two-photon molecular imaging *in vivo*. Hc-red fluorescence protein (Hc-RFP) is a fluorescent protein with an emission peak around 618nm, which was developed by random and site-directed mutagenesis of a non-fluorescent tetremeric chromoprotein from the reef coral Heteractis crispa [3]. With a bright fluorescence and an emission peak longer than 600 nm, Hc-RFP can therefore be considered as one of the most suitable choices for protein localization studies and is selected as our live marker. In this talk, we report that by screening from embryos injected with DNA fragment containing a heart-specific regulatory element of zebrafish cardiac-myosin-light-chain-2 gene (*cmlc2*) fused with a Hc-RFP gene, we have successfully generated a zebrafish line that has strong two-photon red fluorescence (as figure 1 shows) expressed in the heart based on a 1230nm femtosecond light source working in the biological penetration window. Furthermore, with this light source we can also take advantage of the detectable higher-harmonic optical signals, including second-harmonic-eneration (SHG) and third-harmonic-eneration (THG) signals, of which the generation processes leave no energy deposition to its interacted matters due to an energy-conservation characteristic, providing the "noninvasiveness" nature desirable for biological studies [4-7]. In previous experiments, we realized a noninvasive and highly penetrative higher harmonic generation microscopy and the complicated developmental process across a 1.5-mm thick live zebrafish (*Danio rerio*) embryo can be observed clearly *in vivo* without any treatment on the studied live wild-type specimens based on a Cr:forsterite femtosecond laser centered at 1230nm. In this talk we will present the studies of the heart development with the developed transgenetic zebrafish embryos (tagged with Hc-RFP) using two-photon fluorescence microscopy (2PFM) combined with higher harmonic generation microscopy (HOM) [8]. With a 1230nm femtosecond light source working in the biological penetration window, the multi-modality microscopy can offer continuous noninvasive in vivo molecular, morphologic, and structural imaging of the developing

heart deep inside the live Hc-RPF transgenetic zebrafish embryo with a high 3D resolution without removing the chorion.

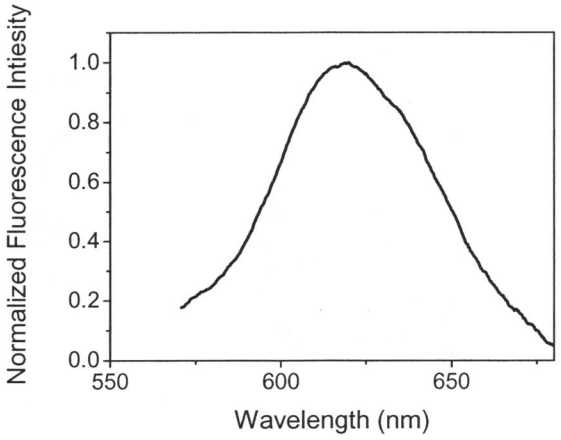

Fig. 1. Two-photon fluorescence spectrum excited with a 1230nm femtosecond Cr:forsterite laser from Hc-RFP in cardiac cells of a live zebrafish. The emission peak wavelength is located around 618nm which matches the emission peak wavelength under single-photon excitation.

Zebrafish has become the standard verterbrate model for the study of the heart development because: (1) zebrafish comes with 1 atrium and 1 ventricle of heart, suggesting a primitive form of the heart of mammals that have 2 atria and 2 ventricles; (2) its heart is completely developed within 2 days after fertilization; (3) in the early stage of the embryo, it can survive with diffused oxygen from water, even the cardiac and vessel system becomes defective, making the study on mutants with a defective heart possible; and (4) mutants can be screened by a simple haploid mutation method [9]. Based on the highly penetrative Cr:forsterite laser light and with the developed transgenic zebrafish line tagged by Hc-RED, the multi-modality nonlinear optical microscopy (2PFM combined with HOM) thus allows noninvasive observation of the heart development deep inside the zebrafish embryos with a sub-micron 3D spatial resolution. The dynamic formation of heart can be easily traced with the aid of the Hc-RFP tag.

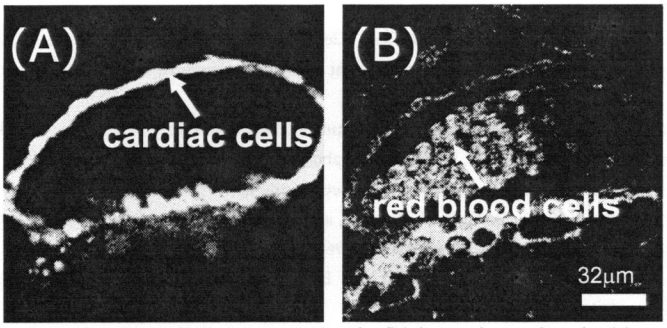

Fig. 2. Cross-sectional *in vivo* images of a live transgenetic zebrafish heart taken at the prim-16 stage, about 30 hours post fertilization, without removing the chorion. A: sectioned image corresponding to the two-photon red fluorescence. B: sectioned THG image.

By minimized the unwanted light-tissue interaction with a 1230 nm light source, the developed multi-modality nonlinear optical microscopy allowed us to observe 3D heart development continuously for a long period of time inside the transgenetic zebrafish embryo *in vivo*. Figure 2 shows example *in vivo* cross-sectional scanning images of the live zebrafish heart at 30 hours post fertilization (30 hpf, at the prim-16 stage) corresponding to the two-photon fluorescence (2PF) and THG modalities respectively . Without removing the chorion, cardiac cells and red blood cells deep inside the live embryo can all be readily observed by the two-photon red fluorescence signal and the THG signal. With the molecular information provided by the red fluorescence signal from the zebrafish cardiac cells, we were thus able to trace the early-stage heart development *in vivo* with a sub-cellular 3D resolution. Figure 3 shows another example *in vivo* cross-sectional scanning images at the depth of the heart surface in the same live zebrafish embryo. With the unique capability

to reflect the distribution of cardiac muscle fibers with SHG, the 3D development of cardiac muscles can be easily studied with this multi-modality microscopy. Furthermore, No optical damage can be observed after long-term continuous observations even with 100-mW incident average power onto the same live embryo. The observed developmental dynamics will be presented in the conference.

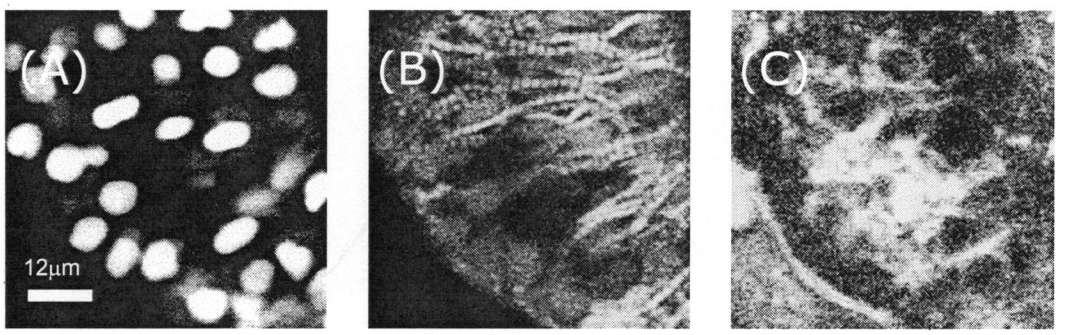

Fig. 3. High resolution cross-sectional *in vivo* images of the heart surface of a live transgenetic zebrafish taken at the prim-16 stage without removing its chorion. A: sectioned image corresponding to the two-photon red fluorescence. B: sectioned SHG image. C: sectioned THG image.. Image contrasts in A, B and C correspond to nuclei of cardiac cells, cardiac muscle fibers, and cardiac cell membranes respectively.

In summary, we have successfully developed a zebrafish line tagged with Hc-Red expression in the heart. Efficient 2PF from the Hc-Red can be excited with a 1230nm femtosecond light source working in the biological penetration window. With a multi-modality microscopy combining 2PF with higher harmonics signals, molecular information (2PF), morphologic information (THG), and protein structural information (SHG) of a developing heart can all be noninvasively revealed deep inside the live embryo with a sub-micron spatial resolution without removing the chorion. The dynamic formation of the heart is successful traced in vivo. With high penetration, high viability and multiple imaging modalities, this new method provides superb imaging capability compared with the traditional GFP-based transgenetic animals, offering deep insight into gene expression in vertebrate embryos.

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