Wide field intravital imaging by two-photon-excitation digital-scanned light-sheet microscopy (2p-DSLM) with a high-pulse energy laser

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Abstract: Digital-scanned light-sheet microscopy (DSLM) illuminates a sample in a plane and captures single-photon–excitation fluorescence images with a camera from a direction perpendicular to the light sheet. This method is potentially useful for observing biological specimens, because image acquisition is relatively fast, resulting in reduction of phototoxicity. However, DSLM cannot be effectively applied to high-scattering materials due to the image blur resulting from thickening of the light sheet by scattered photons. However, two-photon–excitation DSLM (2p-DSLM) enables collection of high-contrast image with near infrared (NIR) excitation. In conventional 2p-DSLM, the minimal excitation volume for two-photon excitation restricts the field of view. In this study, we achieved wide-field 2p-DSLM by using a high–pulse energy fiber laser, and then used this technique to perform intravital imaging of a small model fish species, medaka (Oryzias latipes). Wide fields of view (>700 μm) were achieved by using a low–numerical aperture (NA) objective lens and high–peak energy NIR excitation at 1040 nm. We also performed high-speed imaging at near-video rate and successfully captured the heartbeat movements of a living medaka fish at 20 frames/sec.

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Fluorescence microscopic techniques are widely used in biological studies as practical tools for visualizing the locations and functions of molecules in cells and tissues. When used in combination with fluorescence proteins or synthetic fluorescent dyes, laser scanning confocal microscopy (LSCM) enables collection of optically sectioned high-resolution images of biological samples. Optical sectioning is possible because LSCM uses a detection pinhole that rejects photons emitted outside the focal plane. Although LSCM can provide three-dimensional images of cultured cells or regions near the surface of a tissue, photobleaching and phototoxicity often become significant problems due to out-of-focus excitation. In addition, the penetration depth of biological tissue is restricted by scattering and absorption of the incident light. Nonlinear optical imaging techniques, such as multiphoton-excitation fluorescence microscopy (MPEFM), are promising tools for intravital imaging in deeper regions of biological specimens [1–4]. MPEFM reduces not only photobleaching of fluorophores and phototoxicity toward living cells, but also scattering and absorption of the excitation light, for two reasons. First, multiphoton absorption occurs only at the focal point, avoiding out-of-focus excitation. Second, the illumination light used for multiphoton excitation is relatively long-wavelength, in the near infrared (NIR) region. Nevertheless, for repeated imaging and collection of multiple stacks from a single specimen, longer periods of image acquisition are required, and photobleaching and phototoxicity can affect the specimen even in MPEFM [5, 6].
To address these issues for bioimaging applications, alternative optical sectioning techniques such as selective plane illuminated microscopy (SPIM) and digital scanned light-sheet microscopy (DSLM) have been developed [7–11]. In SPIM and DSLM, the excitation laser beam is irradiated on the focal point of the objective in a perpendicular sheet. Thus, SPIM and DSLM reduce photobleaching and phototoxicity relative to conventional wide-field fluorescence microscopy, LSCM, and MPEFM. In SPIM and DSLM, image acquisition is performed with highly sensitive high-speed imaging devices such as charge-coupled device (CCD) cameras and complementary metal-oxide semiconductor (CMOS) image sensors, and is therefore much faster (30–200 frames/s) than laser-scanning techniques such as LSCM and MPEFM [12]. However, the scattering of illumination affects image contrast and axial resolution in SPIM and DSLM, due to out-of-focus excitation and thickening of the light sheet by scattered illumination light [13].

The combination of light-sheet illumination techniques and multiphoton-excitation fluorescence can improve image quality and penetration depth in biological tissues. Development of two-photon–excitation SPIM or DSLM (2p-SPIM or 2p-DSLM) has been reported by several research groups [14–16]. Truong et al. demonstrated that for imaging of fly embryos, the penetration depth of 2p-SPIM is about twice as high as that of single-photon–excitation SPIM (1p-SPIM) [13]. On the other hand, fields of view more than 1 mm wide can be imaged in conventional 1p-SPIM [7], whereas in the 2p-SPIM system, the maximum field of view is less than 250 µm, even though bidirectional illumination was used [13].

To obtain sufficient photon density for multiphoton excitation with a low–numerical aperture (NA) objective, it is necessary to use a high-power laser. In this study, we developed 2p-DSLM using a low-NA objective and a femto-second high-energy pulse fiber laser. This method achieved the broadest field of view achieved to date. In general, high-energy excitation light has a detrimental effect on biological samples, but the average laser power used for observation in our 2p-DSLM technique was low enough that we could observe live model fish species. Our setup allowed imaging of a wider two-photon excitation region than any system reported previously, and enabled observation of biological samples without significant damage.

2. Materials and methods
2.1 Light-sheet microscopy system

Our light-sheet microscopy system was designed for two-photon–excitation fluorescence imaging based on the selective plane illumination method (Fig. 1). An NIR ultra–short pulse fiber laser (FCPA μ Jewel D-1000, IMRA America, Inc., Ann Arbor, MI, USA) was employed as the excitation light source for multiphoton imaging [17]. For single-photon fluorescence imaging, a diode laser (85BCD, continuous wave laser, 488 nm, 30 mW, Melles Griot KK, Tokyo, Japan) was also equipped. In the illumination light path, the NIR laser entered an illumination objective lens (plan-NEOFLUAR, × 5, N.A. = 0.15, Carl-Zeiss Co., Ltd., Oberkochen, Germany) through a Gran laser prism (GLP0-08-12AN, Sigma Koki Co., Ltd., Tokyo, Japan) and a half-wave plate (WPQ-10640-2M, Sigma Koki Co., Ltd., Tokyo, Japan), in order to allow adjustment of the illumination laser power by rotation of the half-wave plate. The illumination laser was focused inside the specimen by the illumination objective. A galvanometer scanner (VM500 + , GSI Group Inc., MA, USA) allowed scanning (<200 Hz) of the spherically focused illumination beam along the Y-direction at the specimen, thereby generating a scanned light sheet in the X-Y plane. The specimen was placed in a custom-made chamber with a sample holder that could be moved by motor actuators (M.111-1DG and M.116DG, Physik Instrumente GmbH & Co. KG, Karlsruhe, Germany). Detailed information about the custom-made sample chamber and sample holder was described previously [16]. In the observation light path, an objective lens
(UMPLFLN10IR, × 10, N.A. = 0.3, Olympus Corporation, Tokyo, Japan), emission filters, a tube lens (f = 180 mm, SLB-25.4-180P, SIGMA KOKI Co., Ltd., Tokyo, Japan), and an EM-CCD (ImagEM 1K C9100-14, Hamamatsu Photonics, Shizuoka, Japan) were used for acquisition of both single-photon and two-photon–excitation fluorescence images. For single-photon fluorescence imaging, the same optical setup was used after the dichroic mirror. For two-photon fluorescence imaging, the microscopy system included an IR-cut filter (800SP 2459, Thin Film Imaging Technologies, Inc., MA, USA) and a band-pass filter (BA570-625HQ, Olympus Corporation, Tokyo, Japan).

2.2 Evaluation of light-sheet shape for two-photon–excitation fluorescence imaging

The light-sheet shape was visualized using a fluorescent dye, 0.1% Rhodamine B solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 1% (w/v) low–melting temperature agarose gel (SeaPlaque GTG Agarose, Lonza Inc., Allendale, NJ, USA). Single- and two-photon fluorescence images were collected using the EM-CCD camera with an exposure time of 0.2 sec. The fluorescence images were processed, and the signal intensities were calculated, using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2014).

2.3 Sample preparation

Medaka fish (Oryzias latipes) is widely used as a model system for developmental biology, genetics and biomedical sciences [18]. A transgenic line of medaka fish (Oryzias latipes), d-
rR-Tg (beta-actin-loxP-DsRed2-loxP-GFP) was provided by the National BioResource Project (NBRP) Medaka (http://www.shigen.nig.ac.jp/medaka/). This transgenic line (DsRed medaka fish) expresses *Discosoma* sp. red fluorescent protein (DsRed2) throughout the entire body. Young medaka fish within 7 days after hatching were imaged under anesthesia with 0.01–0.02% w/v tricaine (3-aminobenzoic acid ethyl ester; Sigma–Aldrich Corporation, St. Louis, MO, USA) in tap water. Living medaka fish were embedded in 1% low–melting temperature agarose gel using a syringe, and then submerged in 0.01% tricaine in tap water. To observe anesthetized medaka fish, the syringe was set into a chamber filled with 0.01% tricaine in tap water. The medaka fish were then fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) solution for 6 hours at 4°C. After fixation, the specimens were stored in PBS at 4°C. For microscopic observation, the fixed specimens were embedded in 1% low–melting temperature agarose using a syringe in a chamber filled with PBS.

2.4 Observation and analysis

The specimen in agarose was in the chamber at the focal plane of the detection objective, and irradiated with excitation light through the illumination objective. Fluorescence signals were imaged using the EM-CCD camera. Image acquisition of cylindrical agarose gels containing Rhodamine B solution or PFA-fixed samples was performed at 0.2 seconds per frame (sec/frame) over a field of view of 1024 × 1024 pixels, and imaging of live samples under anesthesia was performed at 20 frames per second (frame/sec) over a field of view of 640 × 480 pixels. The pixel size of acquired images was 1.29 × 1.29 µm. Average excitation power was below 10 mW for observation of cylindrical agarose gels containing Rhodamine B solution, and 25 mW for the fixed and anesthetized live samples. All images were normalized for signal intensity. The ImageJ software was used for all image processing and analyses, including calculation of plot profile, three-dimensional stacking of X-Y plane images, and reconstruction of X-Z plane images. Linear interpolation method was applied to measure the beam width on the plot profiles.

3. Theoretical bases

Theoretical calculations of peak power required for two-photon–excitation fluorescence imaging in our system were performed using typical commercially available femtosecond pulse lasers for multiphoton microscopy systems: Chameleon Ultra II (Coherent Inc.), Mai Tai eHP DeepSee, and InSight DeepSee (Spectra Physics KK). Except when stated otherwise, same optical path and laser wavelength (1040 nm) were used for all calculations.

To observe two-photon–excitation fluorescence effectively, sufficient photon density must be provided using an ultrafast pulse laser. In our demonstration, an ultrafast fiber laser (FCPA µ Jewel D1000) was used to provide light-sheet excitation, because the peak power is essential to confirm the efficiency of two-photon–excitation fluorescence. However, it is inconvenient to compare the required peak power to get the same level of fluorescence signal using different commercial lasers. Instead we calculated and compared the averaged power of the lasers, because the averaged laser power is a commonly used parameter for fluorescence image acquisition. When the average laser power at the focal point of the objective was 10 mW, the peak power was calculated to be 283 kW (indicated in the table below by parentheses with an asterisk). The specifications of typical commercially available lasers, pulse duration ($t_{\text{pulse}}$), repetition frequency ($f_{\text{rep}}$), duty ratio ($R$), average power ($P_{\text{aver}}$), and peak power ($P_{\text{peak}}$) are shown in Table 1. The available peak power of these lasers is approximately 30–90 kW, ~150 times lower than that of the FCPA µ Jewel D1000. If these lasers had been used for the demonstration, they would not have been able to provide sufficient output power for the light-sheet microscopy system. The average power values required to achieve a peak power of 283 kW were calculated to be 3.1, 1.6, and 2.7 W, for the Chameleon Ultra II, Mai Tai eHP DeepSee, and InSight DeepSee, respectively (indicated in parentheses with double asterisk). However, the available output powers were much lower.
than the estimated average powers of typical lasers specialized for multiphoton microscopy systems.

Table 1. Specifications of the FCPA µ Jewel D1000 and typical commercially available femtosecond laser systems

| Laser System          | τ \(\text{pulse}\) | \(f_{\text{rep}}\) | \(R\)       | \(P_{\text{ave}}\) | \(P_{\text{peak}}\) |
|-----------------------|---------------------|---------------------|-------------|---------------------|---------------------|
| FCPA µ Jewel D1000    | 353 f sec           | 100 kHz             | 3.5 \times 10^{-8} | 1000 mW (10 mW)* | 14 MW (283 kW)*     |
| Chameleon Ultra II    | 140 f sec           | 80 MHz              | 1.1 \times 10^{-5} | 1001 mW (3.1 W)** | 90 kW (283 kW)*     |
| Mai Tai eHP DS        | 70 f sec            | 80 MHz              | 5.6 \times 10^{-6} | 190 mW (1.6 W)**  | 34 kW (283 kW)*     |
| InSight DS            | 120 f sec           | 80 MHz              | 9.6 \times 10^{-6} | 80 mW (2.7 W)**   | 83 kW (283 kW)*     |

Calculation equivalent: \(P_{\text{ave}} = \tau_{\text{pulse}} \times f_{\text{rep}} \times P_{\text{peak}}\); \(\tau_{\text{pulse}}\): pulse duration; \(f_{\text{rep}}\): repetition frequency; \(P_{\text{ave}}\): average power; \(P_{\text{peak}}\): peak power. \(R = \tau_{\text{pulse}} \times f_{\text{rep}}\); \(R\): Duty Ratio. All values are given at 1040 nm, except for those of the InSight DS, which are given at 1000 nm. *When the average laser power at the sample point is 10 mW, the peak power is calculated to be 238 kW. **Estimated average laser power calculated from the peak power to obtain sufficient photon energy in the case of the FCPA µ Jewel D1000.

4. Results and discussion

4.1 Evaluation of illumination beam properties for two-photon excitation

We constructed our custom-built light-sheet microscopy system for wide-field 2p-DSLM (Fig. 1). As in conventional DSLM, a virtual light sheet was generated by scanning a focusing laser beam up and down, and this light sheet was used for image acquisition. In such an approach, the image quality depends in large part on the properties of the illumination laser beam. To evaluate the beam properties of the excitation laser used for plane illumination, we obtained two-photon–excitation fluorescence images using our light-sheet microscopy system with a laser with high peak power. The excitation laser power was adjusted to avoid fluorescence saturation. First, we demonstrated that single- and two-photon–excitation fluorescence images of fluorescent dye could be captured perpendicularly to the laser beam without scanning (Fig. 2). In the case of single-photon excitation, the intensity distribution of the fluorescence signal overlapped with the excitation beam path because of a linear interaction between the incident photon energy and fluorescent emission (Fig. 2, top part). By contrast, the intensity distribution of two-photon–excitation fluorescence exhibited a completely different pattern relative to that of single-photon–excitation fluorescence (Fig. 2, second top to bottom). This result supports the idea that the effective field of view in 2p-DSLM is narrower than that in 1p-DSLM. In general, the effective field of view in light-sheet illumination depends on the Rayleigh length of the excitation laser beam. Rayleigh length is predominantly determined by NA of the optical system, including the objective lens and the diameter of the incident pupil. We confirmed that elongation of the Rayleigh length, in order to achieve a wide field of view in 2p-DSLM, could be accomplished by decreasing the NA of the incident beam diameter by narrowing the incident pupil from 10 mm to 3 mm in diameter (Fig. 2, second from top and lower).
The intensity distributions of single- and two-photon (1p and 2p) excitation fluorescence signal of Rhodamine B generated by focusing excitation lasers at 488 nm and 1040 nm, respectively. Each fluorescence image was standardized, and images are shown here in pseudo-color. The top pattern shows the 1p-excitation fluorescence image, and the lower patterns show the 2p-excitation fluorescence images obtained with incident pupil aperture (i.e., diameter of the iris diaphragm located in front of the illumination objective pupil) set to 10, 9, 8, 7, 6, 5, 4, and 3 Ø (mm) corresponding to effective N.A. of 0.14, 0.12, 0.11, 0.10, 0.08, 0.07, 0.06, and 0.04, respectively. “Center” indicates the crude focal plane of the illumination objective. The arrow pointing to the left shows the direction of the illuminating irradiation. Scale bar: 200 µm.

The intensity profiles along the X- and Y-axes, calculated from the images of Fig. 2, are shown in Fig. 3(a) and 3(b), respectively. We calculated full width at half maximum (FWHM) of the profiles, as shown in Fig. 3(c) and 3(d), because (as mentioned above) the intensity distribution along the X-axis determines the effective field of view in 2p-DSLM. The FWHM was a sensitive function of the diameter of the incident pupil (Fig. 3(a) and 3(c)). On the other hand, the intensity profile along the Y-axis, which is related to the beam waist size, determines the thickness of the light sheet that corresponds to the optically sectioned Z-axial resolution of the fluorescence images in both 1p- and 2p-DSLM (Fig. 3(b) and 3(d)). The FWHM values along the Y-axis could also be changed by adjusting the incident pupil size. In this illumination system, the incident pupil size was determined by a diaphragm located in front of the objective lens. The minimum FWHM value was 101.5 µm with a diaphragm 10 mm in diameter. When the diaphragm was narrowed to 4 mm, the field of view became congruent with that of single-photon excitation. The size of the field of view improved more than 7-fold (FWHM = 741.7 µm), but the thickness of the optical sections increased only 2-fold (FWHM = 4.6–8.6 µm), relative to the values before the diaphragm was narrowed. This result suggests that enlargement of the field of view may not significantly affect the optical sectioning effect; if so, spatial resolution along the Z-axis also would not significantly worsen. Thus, the width of the field of view trades off with Z-axis resolution. For optimal results, the aperture of the diaphragm should be adapted according to conditions such as sample size, incident laser power, and fluorescence signal intensity during image acquisition. We performed subsequent evaluations and observations of fish specimens using a 4-mm aperture as the optimal condition.
Fig. 3. Signal intensity profiles of single- and two-photon (1p and 2p) excitation fluorescence. (a), (b) Line graphs of the X-axis (a) and Y-axis (b) profiles calculated from the fluorescent beam images of Fig. 2. The X-axes of the graphs indicate the distance from the point with maximal signal intensity in respective images shown in Fig. 2. The signal intensities (i.e., the vertical axis of the graphs) were normalized to the maximum value of each signal. The aperture size of the iris at the back of the illumination objective was changed from 3 mm (N.A. 0.04) to 10 mm (N.A. 0.14) in diameter for the 2p profiles (indicated in color). (c), (d) The bar graphs of the X-axis (c) and Y-axis (d) full width at half maximum (FWHM) of the profiles (a) and (b) for each aperture diameter (mm).

To obtain high-quality images suitable for quantitative fluorescence image analyses, the thickness of the light sheet must be uniform over the field of view. The light sheet is formed by sweeping the laser beam across the field. To estimate the uniformity of the thickness of the light sheet, we measured the intensity profiles along the Y-axes of the images shown in Fig. 2. Figure 4 shows the Y-axis profiles calculated from the single- and two-photon–excitation fluorescence images. In single-photon–excitation, the FWHM value increased with distance from the center (focal point), as shown in Fig. 4(a). In other words, in 1p-DSLM, the thickness of the virtual light sheet increased drastically near the edges of the field of view. The heterogeneity of the illumination light affects not only image brightness, but also image contrast, due to the degradation of optical sectioning performance. Meanwhile, we confirmed that the intensity profile along the Y-axis was also altered, albeit only slightly, as a function of distance from the center of the field of view in two-photon–excitation (Fig. 4(b)). In 2p-DSLM, the thickness of the virtual light sheet was more stable than that of 1p-DSLM along the entire X-axis, from the center (0 µm) to 240 µm away, corresponding to the focal point of
the illumination beam (Fig. 4(c)). This result supports the idea that two-photon–excitation
light-sheet illumination enables collection of obtain homogeneous images by DSLM, even
with a wide field of view.

Fig. 4. The thickness of light-sheet illumination in single- and two-photon (1p and 2p)
excitation. (a), (b) Profiles along the Y-axis, calculated from the 1p and 2p fluorescence beam
images in Fig. 2. Each graph was measured from 0 µm to 240 µm from the center of the beam
images at 40-µm intervals. In the 2p data series, the aperture diameter was fixed at 4 mm (N.A.
0.06). The signal intensities (vertical axes of the graphs) were normalized to the maximum
value of each signal. The horizontal axes of the graph indicates the distance from the center of
the image. (c) Line graphs of the full width at half maximum (FWHM) of the plot profiles in
(a) and (b).

4.2 Two-photon–excitation fluorescence imaging of biological samples

To evaluate the potential of our 2p-DSLM system as a practical tool for intravital imaging, we
performed a demonstration of two-photon–excitation fluorescence imaging by using
transgenic medaka fish. Light-sheet illumination microscopy system is the most suitable
technique for observing embryos and small organs of species such as medaka fish; in the past,
both DSLM and SPIM have been developed and applied for live-cell imaging in the field of
developmental biology [7]. First, we performed fluorescence imaging of fixed DsRed medaka
fish, in which the fluorescent protein DsRed2 is expressed throughout the whole body, by
single- and two-photon–excitation with a scanning illumination beam in our microscopy
system (Fig. 5(a)–5(c)). Figure 5(b) and 5(c) represent single- and two-photon–excitation
fluorescence images of eye and abdomen of DsRed medaka fish at a depth of 143 µm from
the body surface. The structures of some organs (anatomical locations are shown in Fig. 5(a))
were visualized in both images. Using our custom-built microscopy system with a high–peak
power laser, we were able to capture sharp two-photon fluorescence images over a wide field
of view (more than 1000 µm). For image acquisition, the excitation laser power was 30 mW
under the illumination objective, and the frame rate was 5 frame/sec. The intensity of two-
photon–excitation fluorescence is proportional to the square of peak power [1]. Even if
conventional laser sources are used at their maximum output power, it is not possible to
obtain sufficient photon density at the same image brightness in two-photon–excitation
fluorescence, as mentioned in the *Theoretical bases* section of this manuscript. In a previous report of 2p-SPIM with bidirectional illumination, two-photon fluorescence imaging of fly embryos was demonstrated using a conventional Titanium Sapphire laser source (*Chameleon Ultra II, see Table 1*). A sufficient signal level over a useful field of view (approximately 200 µm in the X-axis) was obtained at 200 mW average excitation laser power with a small illumination NA (0.06–0.08) [13]. The useful field of view in 2p-SPIM depends on the focusing properties of the illumination beam and the peak power of the excitation laser. On the basis of Gaussian beam optics, the focusing beam has a hyperbolic shape, and the distance from the beam waist to the place where the spot size increases by a factor of $\sqrt{2}$ is defined as the Rayleigh length. To achieve a wide field of view in SPIM, the Rayleigh length must be large. In general, high-NA objectives are used for MPEFM to condense photon energy, but this decreases the Rayleigh length. In 2p-SPIM and DSLM, the NA of the illumination objective should be given a threshold for two-photon excitation; consequently, the useful field of view is relatively narrow in both conventional 2p-SPIM and 2p-DSLM systems.

![Image](image_url)

**Fig. 5.** Comparison of image quality in the X-Y plane for single- and two-photon (1p and 2p) excitation, using a biological sample (young DsRed medaka fish fixed in 4%PFA). The arrows pointing to the right show the direction of illuminating irradiation. (a) Schematic of the region of interest (ROI) for the fluorescence image. Labels indicate the anatomical locations of tissues in medaka fish (CAU: caudal; D: dorsal; V: ventral; E: eye; ABD: abdominal part; M: muscle; I: intestine). The arrows indicate the illumination direction of the light sheet. (b), (c) 1p and 2p excitation fluorescence images of DsRed medaka fish at a depth of 143 µm from the side surface of the body. Each image was normalized to the maximum signal intensity. Scale bars: 200 µm. (d) Line graphs showing intensity profiles of 1p (red line) and 2p excitation (blue line) along the X-axis, calculated from fluorescence images along the yellow lines in (b) and (c), respectively. The horizontal axis of the graph indicates the X-axial distance from the left edge of the image.

We also compared the image quality, contrast, and resolution between single-photon and two-photon excitation (Fig. 5b and c). In both images, the specimen was illuminated from the left side of the image; consequently, some image degradation is visible on the right side in the field. Furthermore, in single-photon excitation, the image blurred due to scattering of light generated inside the specimen out of the illumination plane (Fig. 5b and c). To quantitatively
analyze the spatial resolution of these images, we plotted the profiles of fluorescence intensity along the X-axis of images obtained by single- and two-photon–excitation, respectively (Fig. 5d). These two graphs depict the body structure of medaka fish in approximately the same position (Fig. 5b and c, yellow lines). In two-photon–excitation, the background level is obviously lower, and the contrast is also higher, than in single-photon–excitation (Fig. 5b and c). As shown in the intensity graphs (Fig. 5d), the background level is relatively low (0–250 and 1000–1100 µm) and the shape of the peaks was much sharper in two-photon–excitation (blue line) than in single-photon–excitation (red line). Thus, in our microscopy system, the spatial resolution in the X-Y plane is improved by two-photon–excitation. In the light-sheet illumination system, the lateral (X-Y plane) resolution is determined by the detection optics and the thickness of the light sheet. Characterization of the beam properties indicated that the FWHM values differed slightly between single- and two-photon–excitation (Fig. 3). Nevertheless, the lateral resolution of 2p-DSLM in the biological sample was superior to that of 1p-DSLM (Fig. 5). These results support the idea that NIR excitation can, in principle, reduce excitation light scattering inside the specimen, which may affect image contrast and resolution in wide-field detection.

In addition, to evaluate the spatial resolution in the axial direction (Z-axis), we performed Z-stack image acquisition and reconstructed X-Z sectioning images for both single- and two-photon–excitation (Fig. 6a–d). Maximum-intensity projection images of single- and two-photon–excitation are depicted in Fig. 6a and c, respectively. The illumination direction is indicated by white arrows. In the two-photon–excitation image, the image contrast is higher than that in single-photon–excitation, and the structures of organs inside the body are accurately reflected in the fluorescence image. The reconstructed X-Z images, along the yellow lines (in Fig. 6a and c), are shown in Fig. 6b and d. In single-photon–excitation, the reconstructed X-Z image was much bluer, but in two-photon–excitation these images were sharp. The profiles of the signal intensity along the Z-axis, lines A and B in Fig. 6b and d, were calculated as shown in Fig. 6e and f, respectively. In the deep portion of the specimen (250–500 µm from the surface), the image contrast of the two-photon–excitation image is higher than that of the single-photon–excitation image (Fig. 6e and f). The image features, as shown by graphs of intensities along the colored lines in Fig. 6b and 6d (blue, line A; green, line B; plotted in Fig. 6e and f, respectively), were sharper in two-photon–excitation than in single-photon–excitation (Fig. 6b and 6d). We also performed three-dimensional rendering of the Z-stack image series obtained by two-photon–excitation (Fig. 6g). The reconstructed X-Z image and the volume rendering image obtained by two-photon excitation closely reflect the structure of deep regions of the tissue (See Media 1). Moreover, the profile along line B, which was located farther from the illumination objective lens than line A, yielded sharper features in two-photon–excitation. In deep regions of a large sample, the axial (X-Z plane) resolution depends not only on the thickness of the light sheet, but also on scattering and refraction of the fluorescence signal from the focal plane inside the tissue. Although the axial resolution worsens as depth increases, two-photon excitation allows relatively high resolution because it enables more effective optical sectioning than single-photon excitation (Fig. 6).
Fig. 6. Comparison of the image quality in the axial direction (X-Z plane) for single- and two-photon (1p and 2p) excitation, using a biological sample (young DsRed medaka fish fixed in 4%PFA). The arrows pointing to the right show the direction of illuminating irradiation. (a), (b) The maximum intensity projection of Z-stacked images generated from 1p and 2p excitation fluorescence images of DsRed medaka fish, collected at intervals of 2.5 μm steps from the side surface of sample. (c), (d) X-Z plane images reconstructed from the Z-stack image of 1p and 2p excitation fluorescence images. Each image was cross-sectioned along the yellow lines shown in (a) and (b). Each picture was normalized to maximum signal intensity. The detection objective lens is the upper side of the image. Scale bars: 200 µm. (e), (f) Line graphs of the Z-axis intensity profiles calculated from the blue line A (e) and the green line B (f) from the images shown in (c) and (d), respectively. The horizontal axis of the graph shows the Z-axial distance from the top edge along each line in images (c) and (d), respectively. (g) Multi-angle viewing of 2p excitation fluorescence images of the DsRed medaka fish obtained by volume rendering of Z-stack images (Media 1).
As mentioned in the Introduction, the speed of image acquisition is the most important advantage of using of DSLM for live imaging. To demonstrate the advantages of fast image acquisition, we performed real-time two-photon fluorescence imaging of live medaka fish (See Media 2). In these experiments, DsRed medaka fish were held in observation chambers under anesthesia. Using two-photon excitation, heartbeat motions were successfully captured at nearly video rates (50 ms/frame; 20 frames/sec), as shown in Fig. 7. In the two-photon–excitation fluorescent images of the tissue surrounding the heart, it is possible to see dilation of the ventricle (c), followed by constriction of the atrium and the bulbus arteriosus (d–g). In the next images, the ventricle of the heart constricts and then dilates again (h–j). No obvious photodamage could be observed during image acquisition. In this system, the repetition frequency is 100 kHz, significantly lower than those of laser sources commonly used in two-photon–excitation fluorescence imaging (generally, 80 MHz, See Table 1). The low repetition frequency of the excitation laser source restricts the frame rate of the image acquisition in LSM (laser scanning microscopy). In general, in LSM multiphoton imaging for acquisition of a 512 × 512 pixel field with 100 kHz repetition, it is possible to achieve a frame rate of 8 sec/frame or more over without image degradation [17]. The pixel dwell time is then assumed to be approximately 30 µsec, at least three pulses per pixel, for the excitation. By contrast, in the demonstration, the galvanometer scanner was operated at 200 Hz to achieve video rate. The excitation laser beam made ten round trips (i.e., scanned 20 times) for acquisition of each frame, corresponding to 250 pulses per scan. The beam width was 8 µm and the scanning distance was up to 1300 µm, eventually one or more pulse could be irradiated over all the field of view. This result suggests that low repetition rate does not affect two-photon–excitation fluorescence image acquisition in a DSLM system using a laser with high peak power. Indeed, such a low repetition rate laser has some advantages for nonlinear excitation boost due to large peak power [19, 20]. We also considered phototoxicity and photobleaching by the intravital imaging in this study. As shown in Fig. 7 and Media 2, photodamage was negligible in short term, because no obvious photodamage and normal heart beating could be kept during image acquisition. In addition, the medaka fish successfully recovered from anesthesia and began to swim normally after the observations. As previously reported, Truong et al tested the low photodamage of 2p-SPIM by long term measurement of the embryonic development using fly embryos [13]. Because photodamage propensity is a critical property of any live imaging modality, long term photodamage examination in our 2p-DSLM system should be done for applications in embryogenic studies.
5. Conclusion

We have developed a wide-field high-speed imaging system based on light-sheet microscopy in combination with two-photon excitation, and evaluated the capability and modality of our 2p-DSLM system with a high–peak power laser. Using this method, we successfully obtained high-contrast and high-resolution two-photon–excitation fluorescence images of a living transgenic medaka fish with the widest field of view ever reported. In order for the system to be practical, however, several technical challenges remain to be overcome. Degradation of illumination could be improved using the multidirectional selective-plane illumination method [21]. In addition, a combination of structured illumination methods (i.e., Bessel beam [22, 23]) would enable improvement of image quality and expansion of the field of view. In order to generate a large field of view, the thickness of the sheet increase, which means a decrease in resolution. To overcome a decrease in resolution, implementation of unique light sources including Bessel beam. On the other hand, combination of our system and a high-speed image sensor such as CMOS camera would take full advantage of the enlarged field of view, because the excitation laser energy of our system is extremely higher than that of conventional Ti:Sapphire lasers without bidirectional illumination method as compared with previous 2p-DSLM systems [13–16]. Such technical approaches would improve the feasibility of our light-sheet microscopy system.
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