In situ fluorescence imaging of organs through compact scanning head for confocal laser microscopy

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Abstract. We develop a compact scanning head for use in laser confocal fluorescence microscopy for in situ fluorescence imaging of organs. The head, cylindrical in shape, has 3.5 mm diameter and 30 mm length, and is thus small enough to operate in a living rat heart. The lateral and axial resolutions, defined as full widths at half maximum (FWHM) of a point spread function (PSF), measures 1.0 and 5.0 μm, respectively, for 488-nm excitation and 1.0 and 5.4 μm, respectively, for 543-nm excitation. The chromatic aberration between 488- and 543-nm laser beams is well suppressed. We perform Ca$^{2+}$ imaging in cardiomyocytes through the right ventricular chamber of a perfused rat heart in line-scan mode with 2.9-ms time resolution. We also carried out two-color imaging of a fixed mouse heart and liver with subcellular resolution. The compact head of the microscope equipped with a line-scan imaging mode and two-color imaging mode is useful for in situ imaging in living organs with subcellular resolution and can advantageously be applied to in vivo research. © 2005 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.1890411]

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1 Introduction

Laser-scanning confocal fluorescence microscopy has revealed various intra- or intercellular functions, such as Ca$^{2+}$ dynamics, with subcellular spatial resolution.\(^1,2\) Features such as high-speed time-lapse imaging and multicolor imaging are indispensable for elucidating the biological phenomena not only in cultured cells but also in living tissues. Recently, we have established an in situ imaging system for monitoring Ca$^{2+}$ concentration ([Ca$^{2+}$]) in rat hearts\(^2,3\) and have found a variety of abnormal [Ca$^{2+}$] dynamics within the cardiomyocytes on the epicardial surface under normal rhythm. However, it is difficult to image the endocardial surface of the heart in situ, because the chamber of the heart is inaccessible to the bulky microscope. Therefore, a compact and flexible microscope with subcellular resolution is needed for in situ or in vivo fluorescence imaging of living organs.

For flexibility, fiber optics was introduced for delivery of excitation light and the optical signals from the specimen between an objective lens and the bulk optics part of the microscope, since the optical fiber works as a point light source as well as a confocal detector.\(^4\) Since then, several types of compact heads with a single optical fiber\(^5-9\) or an optical fiber bundle\(^10-13\) were developed. The resolution of the single-fiber scheme is known to be superior to that of the fiber bundle, because the fiber bundle limits the lateral resolution due to the limited number of image points, irregular fiber packing, and coupling of the illumination in the neighboring fibers (cross talk).\(^12\) The head of the single-fiber scheme is, however, larger than one of the fiber bundles relaying the image at the distal end, because the fiber bundle does not require a mechanical scanner in the head. No compact heads have afforded high-speed imaging with millisecond time resolution and multicolor imaging, although resolutions of 1 μm have been attained with the single-fiber scheme\(^6-9\) and of 3 μm\(^11,12\) with a fiber bundle scheme. Imaging with subcellular resolution in living organs has not been achieved with the compact heads either.

2 Methods and Materials

2.1 Compact Scanning Head

In this research, we employed the single-fiber scheme for 1-μm spatial resolution and a unimorph-scanner-incorporated compact head\(^6\) for implementing high-speed line scan and multicolor imaging. The apparatus that we have constructed is shown in Fig. 1(a). The compact head used to approach the site of interest has a cylindrical metal cover sleeve with 3.5 mm diam and 30 mm length, and is attached to a metal box containing an optical fiber. The head is small enough to access...
the right ventricular chamber of the rat heart through an
atrium [Fig. 1(b)]. The schematic cross section of the head
shows the main components [Fig. 1(c)]. An aspherical ob-
tective lens (NA = 0.55) and two piezoelectric unimorphs
operating together as an xy scanner are incorporated inside
the metal cover sleeve, and these are packed with a cover
glass at the tip of the sleeve, for use under water-immersion
conditions. The unimorph moves a tube holding the ob-
tective lens and the tip of the fiber, and operates at a resonance
frequency of 350 Hz. Consequently, the time resolution in line-scan
imaging mode is 2.9 ms. The head is connected to the bulk
optics part of the microscope via the optical fiber [Fig. 1(d)].
The typical view field is 90×150 μm with a frame rate of 2
Hz.

2.2 Bulk Optics of the Microscope
The microscope is equipped with an Ar laser at 488-nm
wavelength and a He-Ne laser at 543-nm wavelength for ex-
citation of fluorescence. These laser beams are coupled with a
dichroic mirror. The beam to excite the fluorescence is de-
ivered through an optical fiber with 8.3-μm core (SMF-28,
Coming Incorporated). The end of the fiber works as both a
point light source and a pinhole for the confocal system.

The fluorescent light in the specimen is collected by the objective
lens onto the end of the fiber and is delivered through the
optical fiber. Then the signal is detected with a photomulti-
plier tube after proper filtering for cutting unwanted light,
such as reflectance and scattering of excitation laser or the
fluorescent signals from other dyes when multifluorescence
staining is used.

2.3 Langendorff-Perfused Rat Heart
The heart was excised from a male Wistar rat (9 weeks old)
under anesthesia with pentobarbitone sodium according to the
Langendorff model at a pressure of 100-cm H₂O for 5 min
through the aorta. The perfusion was conducted at 20 °C with
a HEPES-buffered Tyrode’s solution composed of (in mM)
NaCl, 140; KCl, 4.0; MgCl₂, 1.0; CaCl₂, 1.0; HEPES, 20;
and glucose, 10 (pH=7.4 adjusted with NaOH) under oxygen-
ation with 100% O₂. After washout of the blood for 10 min,
the heart was perfused with (Ca²⁺-free) Tyrode’s solution
containing a fluorescent Ca²⁺ indicator, Fluo-3/AM, at rela-
tively lower temperatures (19 to 21 °C for 45 min). The Fluo-
3/AM loading was followed by perfusion with 0.5-mM Ca²⁺
Tyrode’s solution at 35 to 37 °C for deesterification of the
intracellularly loaded Fluo-3/AM (for 10 min). During the
experiment, the heart was perfused with Tyrode’s solution
containing cytochalasin D of 50-μM concentration (033-
17563, Wako).

2.4 Immunostaining of the Heart and Liver
The heart and liver were excised from a mouse (B6;129-Gja,
wild type) under anesthesia with pentobarbitone sodium in-
jected intraperitoneally. After washout of the blood with phos-
phate buffer solution (PBS), the heart and the liver were fixed
with 4.0% paraformaldehyde for 2 h, followed by permeabilization with 0.5% Triton X-100 for 30 min. Thereafter, the organs were incubated in Alexa Fluor 488 phalloidin (A12379, Molecular Probes, Tokyo, Japan). Incorporated for 2 h and in propidium iodide (P5264, Sigma Chemical Company, Tokyo, Japan) for another 30 min. To specify the nuclear staining with propidium iodide, the organs were equilibrated in 2X saline-sodium citrate (SSC; NaCl, 0.3 M; sodium citrate, 0.03 M, pH 7.0) for 1 min, incubated in 100-μg/mL DNase-free RNase in 2X SSC for the following 20 min at 37°C, and rinsed with 2X SSC. During experiments, the heart and liver were immersed in PBS.

3 Results and Discussions

3.1 Spatial Resolution

Since the whole imaging system with the tube at the tip of the fiber is moved for the focus scanning, the optical axis of the beam emerging from the optical fiber is always kept on the axis of the objective lens, even during scanning. We therefore need to concern ourselves only with on-axis aberrations, namely spherical and chromatic aberration, to focus the laser beams tightly with the same focal length as well as to collect the fluorescent light at the specimen to the pinhole detector of the fiber effectively. The aspherical lens has a small amount of wavefront aberration (0.017, 0.011, and 0.005 waves at wavelengths of 488, 543, and 656 nm, respectively, expressed as root mean squares). Figure 2(a) shows the lateral PSF of the microscope, measured as the fluorescence intensity distributions of single 100-nm fluorescent beads placed on a glass substrate. (×) and (○) show the PSFs obtained with the excitations by the 488- and 543-nm laser beams, respectively. From the full width at half maximum (FWHM) of the PSF, the lateral resolutions are determined to be 1.0 μm for both 488- and 543-nm excitation. Figure 2(b) shows axial PSFs, measured as the response of the reflection from the mirror moving along the optical axis. From the FWHM, the axial resolutions for 488- and 543-nm beams are determined to be 5.0 and 5.4 μm, respectively. By use of the aspherical lens, the axial resolution is improved to a greater extent than the lateral resolution compared to the use of a hemispherical lens.6 The chromatic aberration is compensated by the cover glass at the tip of the head, since the cover glass is made of a high-dispersive material, while the objective lens is made of a low-dispersive material. Figure 2(b) shows that there is about 0.5-μm difference of the focal position (the location of the reflection peak) between the 488- and 543-nm laser beams. This is coincident with the numerical analysis, based on a ray tracing method, predicting that the cover glass suppresses 7 μm of the focal position difference to 0.52 μm. We utilized the glass plate in place of a concave lens, which is generally used for chromatic compensation, because of practical considerations involved in assembling the miniaturized components.

It is, however, shown that the glass plate composed of high-dispersive material located in the focusing beam is still effective for the chromatic compensation. The working distance of the compact head, defined as the distance from the cover glass to the focus, is about 200 μm.

3.2 Ca2+ Imaging in Cardiomyocytes of a Perfused Rat Heart

The compact head can access the apex of the right ventricular chamber of a Langendorff-perfused rat heart [Fig. 1(b)]. We visualized changes of calcium concentration ([Ca2+]i) in the subendocardial myocytes with the fluorescent Ca2+ indicator Fluo-3. The fluorescence intensities from the cardiomyocytes provided detailed information about the spatial aspects of Ca2+ dynamics in the myocytes. In a representative set of the

![Fig. 3 In situ images of [Ca2+]](images)

Fig. 3 In situ images of [Ca2+]i in cardiomyocytes. (a) Three sequential xy images of [Ca2+]i, detected in cardiomyocytes at the same position. Under attenuation of the mechanical motion of the heart with cytochalasin D, a fixed region of interest was irradiated with an excitation laser (488 nm). The scale bar denotes 10 μm. (b) Schematic cell shapes and locations. The [Ca2+]i fluctuates in four cells (illustrated in gray) and remains at a constant high level in the black cell. (c) A line-scan (x-t) image. The area corresponding to the dashed line in the schematic (b) is imaged with 2.9-ms time resolution. The abscissa denotes the position of the scanning line, and the ordinate, the temporal axis. (d) The line plots of the [Ca2+]i changes along the solid line (1) and the dashed line (2) in (c).
sequential xy images of \( [Ca^{2+}] \), detected at the same position [Fig. 3(a)], three different images revealed localized fluctuations of \( [Ca^{2+}] \), which enabled us to recognize the shapes of the myocytes [Fig. 3(b)]. However, such fluctuations may not reflect the precise behaviors of the myocytes, because the image acquisition time (0.5 s) was too slow to acquire the \( [Ca^{2+}] \) dynamics, such as \( Ca^{2+} \) transients and \( Ca^{2+} \) waves, on a millisecond time scale. To detect the \( [Ca^{2+}] \) dynamics with higher time resolution, we imaged myocytes with the line-scan mode that allows shorter time resolution of 2.9 ms. The line-scan image scanned along the dashed line shown in Fig. 3(b) revealed that the \( [Ca^{2+}] \) fluctuated in two cardiomyocytes periodically and asynchronously to each other [Fig. 3(c)]. The corresponding line plots [indicated by the dashed line in Fig. 3(d)] clearly demonstrated that these two cells fluctuate at frequencies of 1.8 and 2.1 Hz.

3.3 Two-Color Imaging of Fixed Organs

Two-color imaging provided precise depiction of subcellular structures within the whole fixed organs. As shown in Fig. 4(a), in the myocytes on the subepicardial surface of a mouse heart, the actin filaments and nuclei were clearly visualized by immunostaining with Alexa Fluor 488 phalloidin and propidium iodide, respectively. The cross striations were finely aligned at almost regular intervals of around 2.6 \( \mu m \). The nuclei in the cells were clearly distinguished from the cytosol. The cell structure and nuclei of the hepatocytes on the surface of the liver were also visualized [Fig. 4(b)]. These observations confirm that the two-color imaging provides a high spatial resolution for subcellular structures in the organs. Though the images are sliced or sectioned with 5.0 or 5.4 \( \mu m \) thickness, we cannot obtain consecutive sectioning images in depth because of the lack of scanning system in depth. These images were obtained with a frame rate of 2 Hz.

4 Conclusion

We successfully develop a compact scanning head for confocal laser microscopy with lateral resolution of 1.0 \( \mu m \) that affords high-speed line-scan imaging and multicolor imaging. The head is small enough for imaging the living heart. This compact microscope system should potentially be a powerful tool not only for basic biological research but also for medical applications, such as in vivo diagnosis with endoscopic usage. 14

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