Confocal Laser Scanning Microscopy Based on a Silicon Photomultiplier for Multicolor In Vivo Imaging in Near-Infrared Regions I and II

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1. Introduction

To date, confocal laser scanning microscopy (CLSM) has become a fundamental imaging tool in biomedical fields,[1,2] enabling the visualization of cellular and molecular processes.[3–5] In particular, CLSM relies on point-by-point illumination and conjugate pinhole to eliminate light signals outside the focal plane to hit the detector, making it an excellent candidate for optical sectioning with higher resolution and signal-to-noise ratio (SNR) as compared to that from a wide-field microscopy.[6] However, most of the presently available systems for CLSM can only image the cells and thin sections of tissue in the visible region or the first near-infrared (NIR-I) window (700 and 900 nm), in which the tissues exhibit strong optical scattering and autofluorescence.[7–9] By contrast, in the near-infrared II (NIR-II, 900–1700 nm) window,[10–12] CLSM systems are expected to exhibit a better imaging performance in the second near-infrared (NIR-II) windows with weak tissue scattering and autofluorescence. However, the indium gallium arsenide (InGaAs) detectors currently used for imaging in the NIR-II region are prohibitively expensive, hampering its extensive biomedical applications. In this study, a novel NIR-II CLSM system is developed by using the inexpensive silicon photomultiplier (SiPM) that can perform the multicolor biological imaging in vivo. Using IR-780 iodide as the contrast agent, the NIR-II imaging capability of constructed CLSM is inspected, demonstrating a spatial resolution of 1.68 µm (close to the diffraction limit) and a fluorophore detection sensitivity as low as 100 nm. In particular, it is discovered that the multicolor imaging performance in both NIR-I and NIR-II windows is comparable to those from multalkali and InGaAs photomultiplier tubes. In addition, 3D NIR-II CLSM is also conducted for in vivo imaging of the vascular structure in mouse ear and subcutaneous tumors. To the best of authors’ knowledge, this is the first time that a low-cost detector based on a SiPM has been used for microscopic imaging of trailing fluorescence signals in the NIR-II region of an NIR fluorescent probe.

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can perform biological imaging with high spatial resolution and excellent penetration depth due to the weak tissue scattering and autofluorescence.\textsuperscript{[13–16]} Although confocal and two-photon fluorescence lifetime imaging show enhanced imaging capability in the NIR-II region,\textsuperscript{[17,18]} the used Indium gallium arsenide (InGaAs) photomultiplier tubes (PMTs) significantly increase the cost to around tens of thousands of US dollars.\textsuperscript{[18–20]} In addition, InGaAs PMT needs deep cooling during the imaging operation, whose quantum efficiency is only 1–3%. Meanwhile, InGaAs PMT has the disadvantage of being irreversibly damaged by excess incident photons in a brightfield environment.

Silicon photomultiplier (SiPM), a weak light detector consisting of an array of avalanche photodiodes operating in Geiger mode, is smaller, much cheaper, more robust, and quite sensitive to weak light\textsuperscript{[21]} than PMTs, and can also be integrated into the point-scanning system for biological microscopic imaging. Previous studies demonstrated that SiPM showed very similar functions in the visible wavelength range as compared to PMT in coherent anti-Stokes Raman scattering microscopy and two-photon fluorescence microscopy systems.\textsuperscript{[22,23]} Whether SiPM can support high-quality imaging in the NIR-II region is unclear. The wavelength response range of NIR-sensitive SiPM can cover the entire NIR-I region, where many commercially available fluorescent probes such as IR-780, IR-820, IR-800cw, and the clinically approved indocyanine green have their emission peaks; as well as in the NIR-II region up to 1100 nm, where commercial probes described above have relatively strong tailing signals.\textsuperscript{[24,25]} This implies that SiPM may have the potential to enable multi-color CLSM spanning the NIR-I and -II regions.

In this study, a new NIR-II CLSM system was developed that utilizes a cheap detection module (only a few hundred US dollars) customized with a commercially available SiPM chip, which can perform multi-color microscopy imaging with the fluorescent label IR-780. We discovered that a SiPM-based CLSM system can carry out multicolor imaging with better SNR than a multialkali PMT-based one in the NIR-I region or InGaAs PMT-based one in the NIR-II region. More specifically, we first inspected the spatial resolution, sensitivity, and penetration depth of our NIR-II CLSM system by using in vitro tests, and then we demonstrated its potential for in vivo imaging by performing 3D reconstruction of blood vessels in mouse ear and optical sectioning of blood vessels within subcutaneous tumors in mice. To the best of our knowledge, this is the first study that a low-cost SiPM-based detector was used for the construction of a NIR-II CLSM system for mapping the fluorescence signals of an NIR fluorescent probe in the NIR-II region, and for multicolor fluorescence microscopic imaging in both the NIR-I and -II regions. This pilot study demonstrates that SiPM can be an economical and efficient option to set up a point-scanning mode-based fluorescent microscopy system for multicolor NIR-I and NIR-II imaging.

2. Results

2.1. Confocal Laser Scanning Microscopy System

Using a commercially available NIR-sensitive SiPM chip, we designed an appropriate peripheral circuit and power supply to set up a complete detection module (Figure S1, Supporting Information). In addition, all the other components in the microscopy system were obtained commercially (Figure 1a; Figure 1b).
A semiconductor laser with a central wavelength of 785 nm serves as the light source. The incident light is collimated by a lens, reflected by a dichroic mirror (800 nm long-pass, Semorck), and then enters the 2D galvanometer (GVS002, Thorlabs) for planar scanning. Relay lenses expand the laser beam to make full use of the numerical aperture of the objective. Further, the expanded beam enters the objective and is focused on the sample atop a stage that can move in three dimensions. According to the reverse pathway, stimulated fluorescence travels from the sample to the dichroic mirror, which then passes through a converging lens and a fast-switch filter (840 nm band-pass filter for NIR-I imaging, 900 nm long-pass filter for NIR-II imaging), and finally reaches the detector. The defocused fluorescence signal is blocked using a pinhole with a diameter of 100 µm. The detector and optical system are coupled by optical fiber.

2.2. Emission Spectrum of IR-780 Iodide

The emission spectrum of IR-780 was determined using a fluorescence spectrometer. After excitation at 785 nm, the dye emitted at a peak ≈840 nm, with a broad tail extending to 1100 nm (Figure 1b). More than 20% of total emitted fluorescence occurred at wavelengths longer than >900 nm. Of this broad emission spectrum, the multialkali PMT detected emission only in the NIR-I region (<920 nm), while the InGaAs PMT detected emission only in the NIR-II region (>950 nm), completely missing the emission peak at 840 nm. In contrast, the SiPM detected nearly the entire emission spectrum of IR-780 in the NIR-I and NIR-II regions (Figure 1c; Figure S3, Supporting Information).

2.3. Benchmarking the Signal-To-Noise Ratio of the SiPM

The performance of the three detectors was compared using our system and a glass capillary containing 10 µm IR-780. The multialkali and InGaAs PMTs detected signal in accordance with their limited wavelength response range, while the SiPM imaged the sample in both NIR regions (Figure 2). For images of NIR-I CLSM, although multialkali PMT has smaller dark current fluctuation in the background, there is a lot of noise in the signal region. The SNR is only 60% of that of SiPM. For images of NIR-II CLSM, the performance of the SiPM is significantly better than the InGaAs PMT in terms of background noise, image noise, and SNR. The trend of the difference between SNR and the quantum efficiency of the detector is consistent. The detection efficiency of SiPM is approximately three times that of multialkali PMT at 840 nm, and ≈1.5 times that of InGaAs PMT at 1000 nm. Supplementary Figure S4 (Supporting Information) shows the curve of the quantum efficiency of the detectors as a function of wavelength. These results suggest that the SiPM can be a low-cost, high-quality substitute for PMTs in their respective wavelength response.

2.4. Spatial Resolution and Sensitivity of the Silicon Photomultiplier

Using the 1951 USAF target (standard resolution test target developed by the U.S. Air Force in 1951) coated with IR-780 reagent on the back side, we measured a spatial resolution of 1.58 µm in the NIR-I region and 1.68 µm in the NIR-II region (Figure 3a–d), which is consistent with the diffraction limit.
of 1.57 µm. Figure S5 (Supporting Information) shows the resolution when using PMTs, which is consistent with SiPM. These results indicate that the SiPM responds sensitively to fluorescent scintillation in a point-scanning strategy, and that photoelectric conversion is fast enough to avoid obvious artifacts.

Next, we tested imaging sensitivity in the NIR-II region at a weaker luminous flux (10 mW illumination, 10 µs per point). Signal-to-background ratio (SBR) correlated positively with IR-780 concentration below 1 µm, and it was 1.67 at a concentration of 100 nm (Figure 3e,f), suggesting adequate sensitivity for imaging IR-780 in cell cultures or tissues of live animals.

2.5. Analysis of Signal Attenuation with Tissue Depth During Ex Vivo Imaging

To explore the NIR imaging capability of thick tissues, pork slices with various thicknesses were placed on the top of the USAF target and then imaged in the NIR-I and -II regions using our multicolor CLSM system. It was discovered that SBR decreased rapidly with increased pork thickness in both NIR-I and NIR-II regions (Figure 4a) and the ratio varied non-linearly with the imaging depth, showing an inflection ≈150 µm (Figure 4b). NIR-II wavelengths, although lower in energy than NIR-I wavelengths, attenuated with depth much more slowly. As a result, the maximal imaging depth was similar for the
two wavelength regions. Our results are consistent with the idea that imaging at longer wavelengths can compensate for depth-dependent signal attenuation. We found that the SBR remained >1 down to a depth of 480 µm under our experimental conditions (10 µm IR-780, 10 mW illumination, 60 µs per point scanning). However, edges became blurred at this depth, indicating that aberration increases rapidly with depth in this system, reducing the spatial resolution.

2.6. Optical Sectioning and 3D Reconstruction During In Vivo Imaging

We imaged blood vessels in the ear of live mice after injecting the animals in the tail with IR-780 at 3 mg kg⁻¹. To maintain natural tissue tension in the ear during imaging, we did not scan exactly perpendicularly along the tissue surface, such that the axial scanning length did not correspond to the tissue depth. Over an axial scanning range of 600 µm, continuous blood vessels in the ear were clear (Figure 5a; Movie S1, Supporting Information). Images obtained in the NIR-I region were slightly clearer than those obtained in the NIR-II region, reflecting the shallow imaging depth. A good-quality 3D reconstruction of blood vessels (500 × 500 × 600 µm³) was achieved using data from NIR-II images (Figure 5b).

Next, we imaged blood vessels within subcutaneous tumors in live mice. To ensure a flat sample, a coverslip was pressed onto the skin above the tumor. In this case, axial scanning length corresponded to tissue depth. The system was able to image vessels as deep as 120 µm below the skin in both NIR-I and NIR-II regions (Figure 5c). At depths from 0 to 30 µm, good images were obtained in both wavelength ranges. By a depth of 60 µm, however, the lower energy of NIR-II signals meant that NIR-I signals gave better images. At greater depths, the slower attenuation of NIR-II wavelengths meant that images obtained in one wavelength region were similar to those obtained in the other region. Figure S6 (Supporting Information) shows the relative resolution of NIR-I and II images at different depths. The change process is consistent with our in vitro experimental results.

Figure 5. Imaging blood vessels in vivo. a) Representative images of blood vessels in the ear of live mice. Imaging depth is indicated at the upper right. Scale bar, 100 µm. b) 3D reconstruction of blood vessels in mouse ear (500 × 500 × 600 µm³). c) Representative images of blood vessels in tumors in live mice. Imaging depth is indicated at the upper right. Scale bar, 100 µm.
3. Discussion

We have developed a CLSM based on a SiPM that can image in multiple colors spanning the entire NIR-I region and part of the NIR-II region. The SiPM performed comparably to commercially available PMTs in each of those regions separately, offering diffraction-limited spatial resolution and efficient detection of low-concentration fluorophores. Our results demonstrate that SiPM can be used for point-scanning imaging in the NIR, and that the “emission tail” of fluorophores in the NIR-II region can be exploited for high-quality imaging. We further demonstrate the usefulness of imaging in the NIR-II region in order to counteract depth-dependent signal attenuation. In these ways, we prove the application potential of SiPM in all point-scanning imaging systems in the near-infrared region.

An important advantage of SiPM is that it has a response in the whole NIR-I region and part of the NIR-II region, and its detection efficiency is comparable to PMT, which represents support for NIR-I and NIR-II multicolor imaging. Coincidentally, the spectral energy of many commercial NIR fluorescent probes is abundant in 900–1100 nm, while their main emission peak is located in NIR-I. Therefore, the SiPM-based scheme can realize NIR-I/NIR-II multicolor imaging of commercial NIR fluorescent probes with low cost and high efficiency, which can promote the popularization of NIR-II imaging technology. Unfortunately, the spatial resolution and SBR with the SiPM were lower in the NIR-II region than in the NIR-I region during our in vivo imaging experiments. We suspect this reflects the much weaker emission of IR-780 in the NIR-II region than in the NIR-I region. Furthermore, the CLSM in the NIR-II region did not achieve a better imaging depth. In addition to the lower spectral emission energy of the dye in the NIR-II region, the characteristics of the CLSM also contribute to this result. Due to the presence of the conjugate pinhole, the CLSM only detects the fluorescence signal excited within the focused Airy spot of the excitation light, so the geometry and energy density of the focused spot contribute significantly to the imaging quality. This also means that in a confocal scheme the wavelength of the excitation light has a more pronounced effect on the imaging depth than in wide-field fluorescence imaging. In other words, the wavelength of excitation light determines the imaging depth of the CLSM. We plan to validate the detection depth of our system with other NIR dyes and longer wavelengths of excitation light in future work.

We also plan several improvements to the system, such as making the detection module more modular and adding cooling, which helps reduce measurement noise due to temperature variations. The current CLSM system needs to be coupled to the SiPM detector via an optical fiber, which is for compatibility with the scheme of PMTs. However, the physical properties of the SiPM allow it to be integrated into the system without the use of optical fibers, allowing direct detection of spatial light, which can reduce photon losses and improve detection efficiency. Making the pinhole smaller while maintaining luminous flux may help ensure good spatial resolution independently of the emission wavelength. With the current system, spatial resolution was worse in the NIR-II region than in the NIR-I region, and it did not reach the diffraction limit. This likely reflects that we used a larger pinhole in order to increase luminous flux. Our system may also be improved by combining it with stimulated emission depletion or super-resolution optical fluctuation functionality, which would also provide richer biological information.

It should be pointed out that our present system can only provide an imaging depth of less than 200 μm when the SBR is >1.5, which should be further improved to analyze more interesting biological processes in cells and tissues. It might be possible to deepen penetration by replacing the Gaussian beam with non-diffracting Airy or Bessel beams that can recover their shape after being obstructed. In addition, the wide wavelength response range of the SiPM makes it compatible with two-photon imaging at long wavelengths, allowing greater penetration. On the other hand, adding adaptive optics to the system may counteract depth-dependent aberration and resolution loss. Using the broadband response of SiPM in the NIR region, optical microscopy with depth-resolved capability will be a new tool for deep volumetric imaging, such as in vivo brain imaging. In addition, the use of SiPM as a detection unit in single-pixel imaging systems will break the field-of-view limitations of microscopic imaging and enable whole-body fluorescence imaging of small animals.

4. Conclusion

We developed a CLSM system based on a SiPM that can achieve multicolor biological imaging simultaneously in the NIR-I and NIR-II regions. We demonstrate that the system retains the high resolution and sensitivity of traditional PMTs-based confocal microscopy imaging systems. Meanwhile, we illustrate that the system can carry out in vivo imaging, allowing for good-quality 3D reconstruction and optical sectioning. Therefore, the potential of SiPM in all point-scanning imaging systems was determined by both in vitro and in vivo tests, and the foundations laid here may help open up new possibilities for in vivo microscopy in the NIR region.

5. Experimental Section

System Configuration: Starting from a commercially available SiPM chip (MICRORB-10035-MLP, Onsemi), we designed and fabricated a peripheral circuit and power supply to create a detection module (Figure S1, Supporting Information) were designed and fabricated. The reverse bias voltage of the SiPM was typically set to 32 V during operation. In the SNR comparison experiment, it was also used a multialkali PMT with a modular design (PMT1001/M, Thorlabs, an 11 000 V A−1 amplifier was integrated inside the module), for which the gain during the operation was set to maximum in the manufacturer-supplied software; and an InGaAs PMT (HT12397-75, Hamamatsu), which was used in combination with an amplifier (C3319, Hamamatsu, 1 V μA−1) and operated at its maximum voltage of 900 V.

All these detectors sent optical signals through an FC/PC fiber and voltage signals through a BNC jumper to a data acquisition card (PCIe-6363, National Instruments). A 10X magnification objective was used with a numerical aperture of 0.25 (RMS10X, Olympus) with a long working distance in order to reduce the effects of sample thickness and refractive index on imaging.
In vitro Experiments: For all in vitro experiments, IR-780 was dissolved in DMSO. The emission spectrum of IR-780 was determined using a fluorescence spectrometer (FSL-1000, Edinburgh Instruments). In the SNR comparison experiment, IR-780 of 10 µm was placed in a capillary glass tube with a diameter of 300 µm, the illumination power was 10 mW, and the scanning rate was 10 µs per point. In spatial resolution tests, 10 µm IR-780 was applied to the surface of the 1951 USAF resolution test target (R3L31ST, Groups 6 and 7, Thorlabs), covered with a coverslip, then inverted before imaging with an illumination power of 10 mW and scanning rate of 10 µs per point. In experiments to analyze signal attenuation with imaging depth, pork meat without visible fat was frozen at −20 °C, then sliced to thicknesses of 80–560 µm. The pork was placed on top of the resolution test target, on the side facing the objective, and imaging was carried out with an illumination power of 10 mW and a scanning rate of 60 µs per point.

Data Processing: All fluorescence imaging was carried out using in-house control and acquisition programs running in LabVIEW 2017 (National Instruments). The lateral scanning step size was 1 µm per pixel. Images were drawn using Image J 1.52a and Origin 9.0 (OriginLab). Original images were used to calculate SNR and SBR, as well as spatial resolution. The color range in images was modified for display purposes.

SNR was determined by subtracting the mean value of background noise from the pixel values in five areas of the signal along the diagonal of the image, then calculating the ratio of the mean value to the standard deviation.[32] SBR was calculated as the ratio of the mean pixel value in the signal area to the mean pixel value in the background area, each sample was calculated 5 times and the mean and variance were taken for plotting. Spatial resolution was calculated by extracting the sequence of pixel values at the light-dark interface in the image, calculating the first derivative, and then performing Gaussian fitting. The full width at half-maximum of the resulting Gaussian curve was taken to be the spatial resolution.

Animal Experiments: Animal experiments were performed according to the Ethical Guidelines on Animal Care with approval from the First Affiliated Hospital of Fourth Military Medical University (KY20163349-1). 5 × 10⁶ ECI109 human esophageal squamous cell carcinoma cells in 100 µl of Matrigel (Corning, Tewksbury, MA, USA) were subcutaneously inoculated near the right hindlimb of 4-week-old female BALB/c nude mice. Tumors were allowed to grow, and their size was measured using Vernier caliper. Animals were anesthetized, then injected intravenously with IR-780 in phosphate-buffered saline at a dose of 3 mg kg⁻¹. Mice. Tumors were allowed to grow, and their size was measured using Vernier caliper. Animals were anesthetized, then injected intravenously with IR-780 in phosphate-buffered saline at a dose of 3 mg kg⁻¹. Imaging blood vessels in the ear, the ear was in a state of natural extension, the illumination power was 10 mW, the scanning rate was 60 µs per point. In experiments to analyze signal attenuation with imaging depth, pork meat without visible fat was frozen at −20 °C, then sliced to thicknesses of 80–560 µm. The pork was placed on top of the resolution test target, on the side facing the objective, and imaging was carried out with an illumination power of 10 mW and a scanning rate of 60 µs per point.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords
confocal laser scanning microscopy, in vivo microscopy, NIR-II imaging, silicon photomultiplier

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