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Abstract. Multiphoton microscopy has become essential for dynamic imaging in thick living tissues. High-rate, full-field image acquisition in multiphoton microscopy is achievable by parallelization of the excitation and detection pathways. We developed our approach via a diffractive optical element which splits a pulsed laser into 16 beamlets and exploits a descanned detection system consisting of an array of beamlet-associated photomultiplier tubes. The optical performance of the multiphoton multispot system (MCube) has been characterized in cardiac tissue sections and subsequently used for the first time for fluorescence imaging of cardiomyocyte Ca^{2+} dynamics in viable acute cardiac slices. Multispot multiphoton microscopy (MMM) has never been used before to monitor Ca^{2+} dynamics in thick, viable tissue samples. Acute heart slices are a powerful close-to-*in vivo* model of Ca^{2+} imaging allowing the simultaneous observation of several cells in their own tissue environment, exploiting the multiphoton excitation ability to penetrate scattering tissues. Moreover, we show that the concurrent high spatial and temporal resolutions afforded by the parallel scanning in MMM can be exploited to simultaneously assess subcellular Ca^{2+} dynamics in different cells in the tissue. We recorded local Ca^{2+} release events including macrosparks, travelling waves, and rotors. © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: [10.1117/1.JBO.20.5.051016](https://doi.org/10.1117/1.JBO.20.5.051016)]

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1 Introduction

The advent of two-photon excitation microscopy (TPEM) has revolutionized biological imaging by enabling the detection of dynamic cellular processes in intact tissues deep into the organs of living organisms. When combined with the use of fluorescent Ca^{2+} indicators, TPEM can be used to follow the variation of intracellular $[\text{Ca}^{2+}]$ within the cells in their physiologic environment (i.e., the tissue).

In the heart, intracellular Ca^{2+} is a key determinant of cardiomyocyte function, which is regulated by a complex of signals shaped in time (from millisecond to second) and space (from subcellular to cell-wide) within the single cell. In addition, fast and chaotic Ca^{2+} signals occurring during arrhythmias involving, by definition, multiple cells in the tissue add another layer of complexity as cell-cell interactions take part in the arrhythmia pathophysiology. The ideal method to visualize intracellular Ca^{2+} fluctuations in the intact myocardium would thus be endowed with high temporal and spatial resolutions and, preferentially, be able to record the cellular fluorescence from a wide field of view.

Up until now, one of the limitations of conventional TPEM is the difficulty in acquiring full frame (512×512 pixels) images at a frame rate significantly higher than 1 Hz, thus hampering the simultaneous detection of transient dynamic events lasting fractions of a second in a wide sample region.^{1,2} Different

solutions have been used³ to achieve high speed and resolution while retaining the advantages of two-photon excitation (TPE), such as penetration depth and reduced out of focus phototoxicity, with respect to the traditional confocal or fluorescent microscopy.⁴ Reduced scanned area and/or pixel resolution are the straightforward ideas developed by microscopy companies and are normally used by several research groups to increase the image acquisition rate.⁵⁻⁷ However, all these strategies allow faster acquisition at the expense of the spatial details, up to the most extreme case of a single-line scan that uses the deflector to sweep the laser beam along a single line, thus generating two-dimensional (x, t) images. One alternative strategy to achieve full-field imaging at a high rate would exploit faster beam scanners and compensate for the inherent reduction in pixel dwell time by intensifying the incident laser power. However, given that the photodamage of biological specimens excited at two photons increases nonlinearly with the laser power,⁸ this last approach has very little use in living samples. To reduce photodamage and photobleaching, Konnerth and collaborators demonstrated the possibility to shorten the dwell time without compromising the signal using low-power temporal oversampling. In this technique, a mirrorless scanning two-photon microscope is used and has been applied to image Ca^{2+} signals *in vivo*.^{9,10} Other techniques exploiting acousto-optic deflectors instead of galvanometric mirrors have been applied to Ca^{2+} imaging¹¹ but do not solve the problem of high resolution in a large field of view. An alternative and promising approach to image thick samples while maintaining large field of view consists of the combination of TPEM and selective

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plane illumination microscopy. This technique has been used for developmental studies¹² and is fully characterized for highly scattering samples.¹³

To increase the acquisition rate of TPEM, we used a parallel scanning system based on the insertion of a diffractive optical element (DOE)¹⁴ along the incident laser path, splitting the beam into a matrix of identical beamlets with a well-defined spatial distribution. By using such an arrangement, the sample area was divided into a number of contiguous subregions, each of which was simultaneously raster scanned by a single beamlet. Parallel acquisition by an array of detectors, with a geometry corresponding to that of the DOE, allowed the spatial resolution of the detection path and the reconstruction of the full-field sample image. The multiphoton multispot microscopy system described in this work (MCube, developed by light4tech¹⁵) was installed at the Department of Biomedical Sciences and was used to test and compare image quality and point spread function (PSF) with those of a conventional TPEM system.¹⁶ Subsequently, the MCube was used for Ca²⁺ imaging experiments in acute heart slices, allowing the simultaneous detection of subcellular Ca²⁺ dynamics in several different cells in the tissue. The cardiac slices' model was selected as a thick-tissue testing platform for multiphoton microscopy as it combines the preservation of the multicellular myocardial structure with the quasi-bidimensional morphology of the slice.¹⁷

2 Materials and Methods

2.1 Conventional Two-Photon Excitation Microscope

A multiphoton system (Radiance 2100 MP; Bio-Rad Laboratories, UK) was used for the comparison of MCube image quality with respect to an existing TPE microscopy system. The radianc system is equipped with a Tsunami mode-locked, tunable, femto-second-pulsed Ti/sapphire laser (Ti:Sa). The laser output is capable of generating 100-fs pulse trains at a rate of 82 MHz. The microscope (Eclipse E600FN; Nikon) was equipped with a Nikon objective (40×/0.8 W CORR); a direct detection system (Bio-Rad), fitted with a 500LP DC dichroic mirror and an HQ535/50 emission filter (Chroma Technology Corp.), was used for the detection of fluorescence emission signals. The LaserSharp2000 software package (Bio-Rad) was used for data acquisition.

2.2 Acute Heart Slices Cutting and Loading

Acute ventricular heart slices were cut following the protocol described in Ref. 18. Young mice (postnatal day 7 to 10) were sacrificed and the heart was quickly excised and washed in ice-cold Ca²⁺-free Tyrode solution (composition in mM: NaCl 136, KCl 5.4, NaH₂PO₄ 0.33, MgCl₂ 1, glucose 10, hepes 5; pH 7.40 with NaOH). Atria were excised by a cut on the transversal plane, also creating a basis surface for fixing the ventricular portion of the heart to the slicer support with a commercial cyanoacrylate glue. Hearts from young mice were embedded in 4% low-melting agarose (SIGMA-Aldrich) in Tyrode. Transversal slices (450-μm thickness) were cut in ice-cold Ca²⁺-free Tyrode solution with a vibratome (Leica GmbH). After cutting, slices were transferred to room temperature 1-mM CaCl₂ Tyrode for some minutes and then they were maintained in the recovery medium (DMEM/F12 supplemented with 20% Knock-out Replacement Serum, Invitrogen) in a humidified atmosphere

containing 95% O₂ and 5% CO₂ at 37°C for at least 1 h before loading.¹⁹

After recovery, slices were loaded with the Ca²⁺-sensitive indicator Fluo-4AM (5 μM, Invitrogen) for 40 min at 37°C in 1-mM Ca²⁺ Tyrode solution plus 20% Pluronic F-127 (Invitrogen) and 1-μM sulfinpyrazone (SIGMA-Aldrich).

2.3 Imaging and Stimulation

Viable heart slices loaded with Fluo-4AM were transferred on a homemade perfusion chamber on the stage of the two-photon microscope and perfused with a 1 mM Ca²⁺, oxygenated Tyrode solution.

To prevent curling and movement, slices were held down by a homemade platinum holder.

Slices were field stimulated by applying directly under the microscope a voltage difference at the slice sides (5 V/cm); slices were thus electrically paced by applying brief voltage square waveforms (5 ms), with the fixed frequency of 1, 2, or 5 Hz. Changes in Ca²⁺ concentration were observed as variations in the green emission of the sample upon excitation.

3 Results

3.1 Instrument Design

Multispot multiphoton microscopy (MMM) allows the increase of the image acquisition speed several fold when compared with standard laser scanning microscopes. Analogous systems based on a microlens array have already been used to monitor Ca²⁺ dynamics in isolated cardiac cells⁷ but, to the best of our knowledge, MMM has never been employed to image cellular Ca²⁺ in whole hearts or *ex vivo* myocardial preparations.

To obtain an MMM system suitable for high-speed imaging at the cellular level in intact organs, we sought to obtain homogeneous light distribution over the sample, while at the same time, maximize light detection from the whole field of view of the objective. The system design is thus based on a DOE in the excitation light path, a descanned configuration in the emission path, and an array of photomultiplier tubes (PMTs) [Fig. 1(a)]. A Ti:Sa pulsed infrared (IR) laser (<150 fs, 80-MHz pulses, Chameleon Ultra II, Coherent, UK) providing up to 3.6 W of optical power at 785-nm excitation wavelength was used as the excitation light source.

The DOE was inserted along the incident light path to obtain patterned illumination of the sample, obtained by splitting the laser beam into 16 parallel beamlets generating a matrix of 4 by 4 spots regularly spaced in a squared array. Such a configuration, in the preliminary tests, offered the best compromise between the imaging rate and signal-to-noise ratio. Laser power was evenly distributed throughout the spots [within a few percent due to the DOE construction, Fig. 1(b)], thus providing uniform illumination of the entire scanned area.¹⁴ With the laser power at the source of 3.6 W, the power per beamlet measured after the 4×4 DOE resulted in about 45 mW.

The 16 parallel beamlet patterns were deflected along the *x* and *y* axes by galvanometric mirrors, while movement of the excitation plane along the *z*-axis was achieved by shifting the objective with a piezoelectric motor.

Detection of the emitted light is performed with a multianode PMT, (H7546A-20SEL from Hamamatsu), with each detecting element corresponding to a single descanned beamlet. Each PMT has its own electronic board, performing signal amplification,

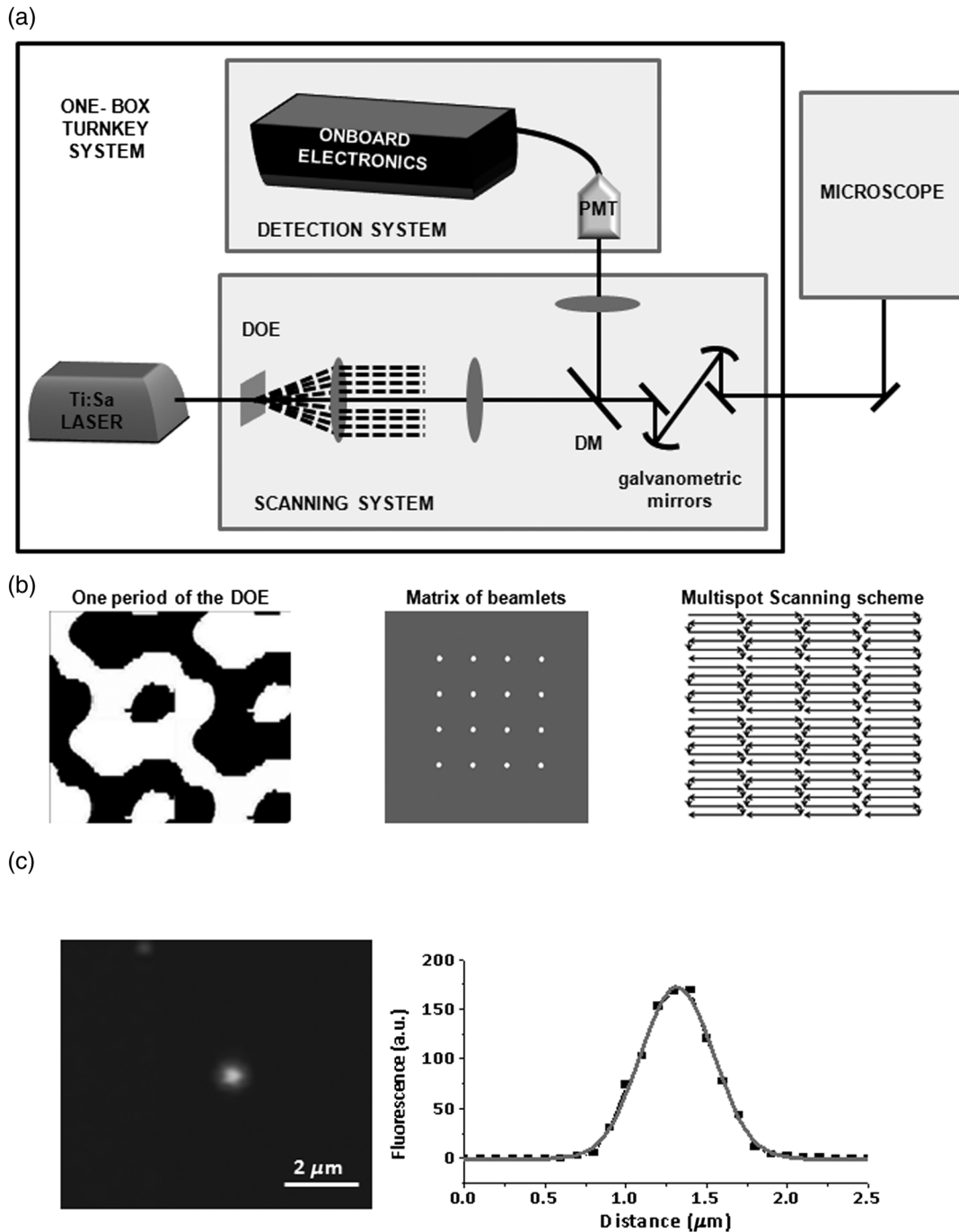


Fig. 1 (a) Scheme of the multispot multiphoton microscope (MMM). (b) Essential components of the MMM. (c) Image and intensity profile of a subresolution bead along the radial axis.

A/D conversion, local storage on a 2 MB onboard memory, and parallel communication with a custom-developed control software for data acquisition and storage on the PC hard drive. This configuration minimizes the optical cross-talk between different PMT elements²⁰ that may introduce undesired degradation of the image quality, particularly when acquiring fluorescence from highly scattering samples such as muscular tissue. The essential components of the MMM are shown in Fig. 1(a).

To allow experiments with living tissue samples, the MMM was adapted to an upright fluorescence microscope (DM LFSA, Leica Microsystems GmbH, Germany), equipped with a water immersion objective (Nikon, 40 × /0.8 W CORR).

3.2 Optical Performance

To evaluate the optical performance of the MMM system, we determined the PSF by collecting the three-dimensional (3-D) image profile of a subresolution fluorescent bead, as shown in Fig. 1(c). From the radial intensity profile in Fig. 1(c), we obtained a radial resolution of 464 ± 10 nm, a value in line with the expected theoretical resolution of 455 nm calculated for a two-photon system at the excitation wavelength of 785 nm.

Imaging of fluorescent beads embedded in agarose has previously been used to measure the PSF of microscopy systems as shown in Refs. 7 and 20. Although such a configuration is

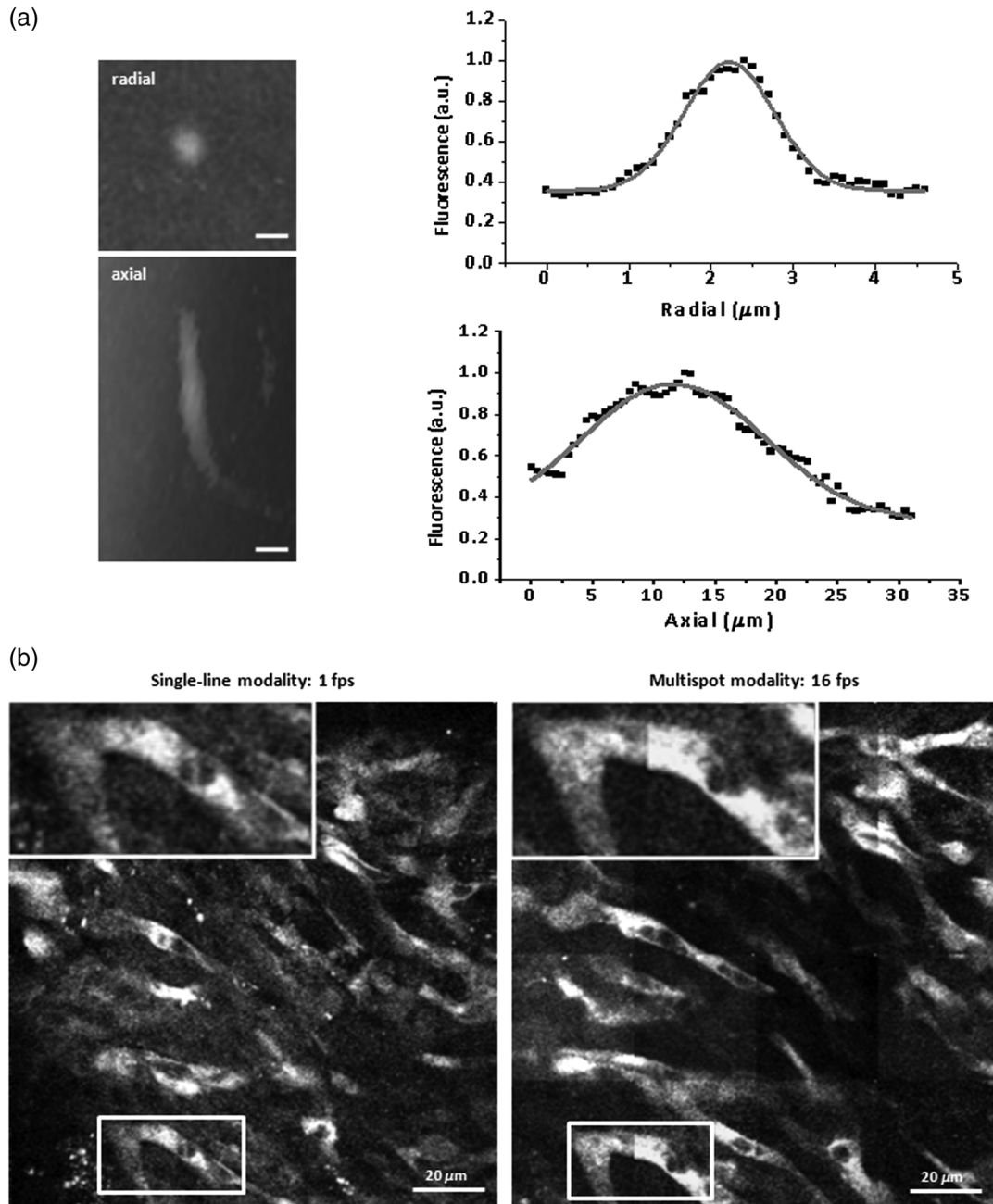


Fig. 2 (a) Intensity profile of a 1-μm fluorescent bead at 150-μm depth in myocardial tissue. Scale bars: 2 μm. Radial and axial views are reported as well as relative intensity profiles. (b) The same image acquired in single-line modality and multispot modality.

adequate for evaluating the pure optical performance of the systems, the sample is almost transparent and isotropic, and thus is not representative of the typical experimental condition. The myocardium is densely occupied by sarcomeres, formed by alternating segments of thin and thick protein filaments arranged in a highly scattering grid, inevitably reducing the performance of an optical system. To test the optical performance of the MMM system in deep tissue imaging in the heart, we evaluated the experimental PSF using a custom-generated heart phantom sample. The phantom was made by injecting 1-μm fluorescent beads (Invitrogen) at different positions and depths in a 500-μm fixed heart slice. We measured the fluorescence profile of a 1-μm bead at a 150 μm depth in the tissue and estimated the system PSF in deep tissue imaging. The ePSF obtained is

Table 1 Full width at half maximum (FWHM) of the measured point spread functions (PSFs) for the single-beam BIORAD MP2100 and multispot MCube systems; data refer to a fluorescent bead located 150 μm in depth in the phantom sample.

	FWHM radial (μm)	FWHM z-axis (μm)
MP2100	2.24 ± 0.14	21 ± 1.2
MCube (with DOE)	1.30 ± 0.05	17.7 ± 0.7

shown in Fig. 2(a) and the characteristics of fluorescence profiles are reported in Table 1.

The results obtained with the MMM were compared with that of a commercial standard, the BIORAD MP2100 multiphoton microscope powered by a Ti:Sa Spectra Physics Millennia pulsed IR laser. We obtained, in both cases, a PSF for TPE microscopy comparable with that reported in other papers,⁷ with a better resolution for the MMM in both the axial and radial directions. The improvement in the optical resolution of the

MMM can be dependent, at least in part, on the spatial filtering of the DOE apparatus. To determine the effect of the DOE insertion, we next compared the quality of the images acquired in either single-line mode or multispot mode of the same field of view with a comparable laser power. In a single-line mode, the laser power was thus attenuated down to 45 mW on the sample plane. As shown in Fig. 2(b), the image resolution and S/N are comparable, demonstrating that the DOE insertion does not cause image deterioration. Notably, the acquisition with the

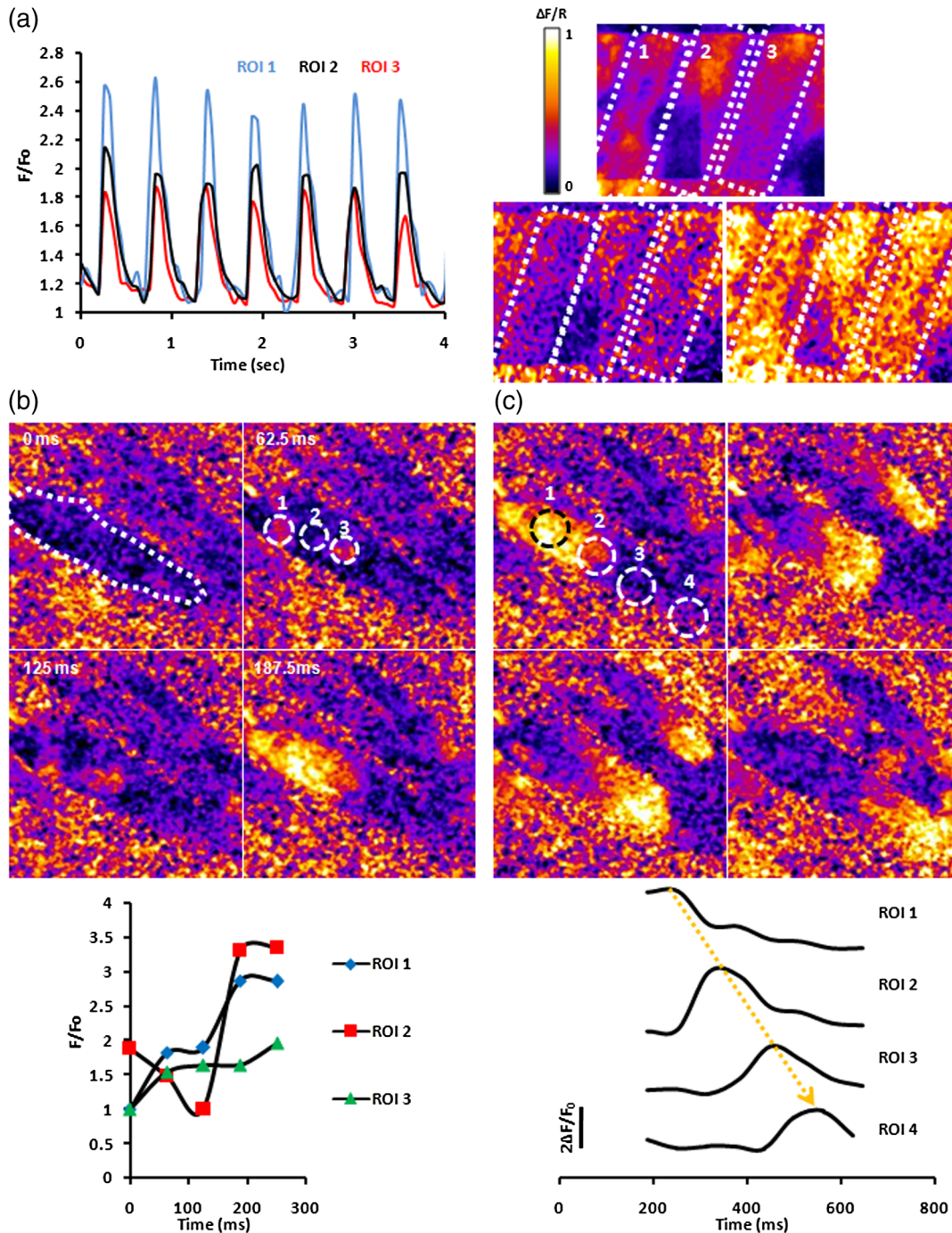


Fig. 3 (a) Intensity profiles of three neighboring cells in the heart slice. Cell borders are highlighted by dotted squares and the fluorescence profiles are reported. It can be seen that cell 2 crosses two sub-frames without affecting temporal fluorescence profiles. (b) Simultaneous onset of macrosparks in two of the three regions of interest (ROI) considered with the respective intensity profiles. (c) A travelling Ca^{2+} wave recorded in the same cell and intensity profiles of the ROIs considered.

DOE inserted is 16 times faster than the single-line modality. In the inset, a magnification is shown.

3.3 Multispot Multiphoton Microscopy Imaging of Calcium Dynamics in Thick Myocardial Slices

The system was tested on viable acute cardiac slices from mouse heart, by performing Ca²⁺ imaging using the loaded fluorescent calcium indicator Fluo-4AM. Acute myocardial slices are an experimental system well representing the intact myocardium, allowing the use of state-of-the-art imaging methods to explore Ca²⁺ signals from the subcellular to the multicellular range.

We used the MMM system to perform two different sets of experiments. In the first, Ca²⁺ imaging was performed at 16-Hz rate during field stimulation in a 137 × 137 μm wide area of the myocardial slice, typically including six to eight cardiomyocytes. Electrical pacing by field stimulation resulted in a synchronous increase in intracellular Ca²⁺ in the cardiomyocytes in the imaging field, as reported in a typical experiment shown in Fig. 3(a). At the acquisition rate used in the experiments, it was possible to fit the Ca²⁺ transients' decay with a single

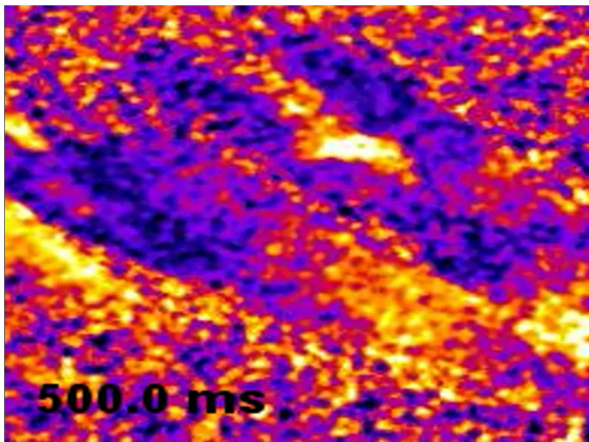
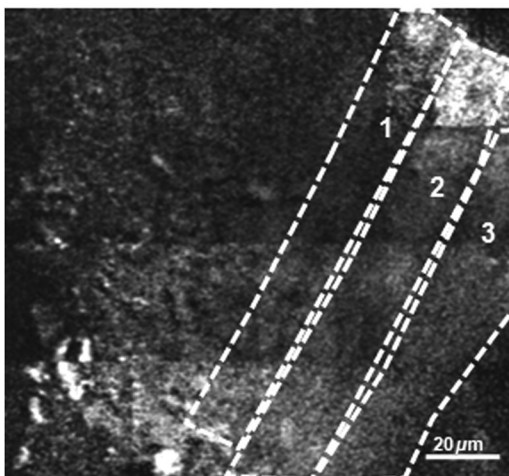


Fig. 4 Travelling Ca²⁺ waves and rotors in the same region of Fig. 3(b) (MOV, 7.93 MB). [URL: <http://dx.doi.org/10.1117/1.JBO.20.5.051016.1>.]



exponential function obtaining a typical decay time in the range of 100 to 150 ms ($n = 14$ cells), in line with data in the literature.²¹ In the subsequent set of experiments, MMM was used to investigate Ca²⁺ dynamics with a subcellular resolution in a networked group of cells in the myocardial slices.

The spatial resolution of MMM at a 16 Hz frame rate was assessed by imaging subcellular Ca²⁺ dynamics in a wide region of the slice. The elementary Ca²⁺ signals regulating the excitation–contraction coupling in heart cells are represented by Ca²⁺ “sparks,”²² i.e., transient and localized releases of Ca²⁺ from the sarcoplasmic reticulum (SR) through the ryanodine receptor Ca²⁺ channels.

A pace-stop protocol was used to increase the frequency of Ca²⁺ release events. Intracellular and local Ca²⁺ release events, both in the form of secondary “macrosparks” [Fig. 3(b)] of approximately 4 to 6 μm in diameter, and self-sustained Ca²⁺ waves traveling through the cell [Fig. 3(c)] could be imaged simultaneously in numerous cardiomyocytes in the slice. Such cell-wide Ca²⁺ waves frequently originated from the same Ca²⁺ release hotspot and, in some cases, were underlined by organized intracellular Ca²⁺ rotors (see Fig 4). Moreover, it was possible to appreciate that the neighboring cells displayed Ca²⁺ waves in a pattern of interdependency (Fig. 5).

4 Discussion

The simultaneous analysis of intracellular Ca²⁺ dynamics in several neighboring cells in their physiologic environment, using fluorescence-based methods, poses a number of technical challenges:

1. A suitable experimental model, with preserved cell viability and intact cell-to-cell interactions in the three dimensions, expressing a fluorescence Ca²⁺ reporter.
2. An optical imaging method, allowing high efficiency in the light collection and high resolution (μm), in an area as wide as needed to include a significant number of cells to detect cellular and subcellular Ca²⁺ variations in multiple cardiomyocytes.

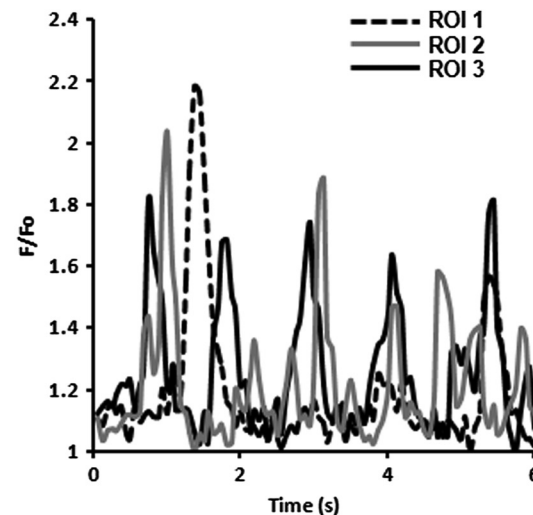


Fig. 5 Three neighboring cells in a large field of view display spontaneous Ca²⁺ releases closely related in time.

3. A system capable of temporal resolution compatible with the dynamics to be investigated.

In standard laser scanning confocal microscopes and TPDM, a single collimated laser beam is scanned on the sample in a raster pattern. The maximal frame rate that can be achieved in such systems is essentially determined by two parameters: (1) the speed of the beam deflectors, typically galvanometric mirrors. (2) the pixel dwell time, determined by the shortest time necessary to achieve sufficient excitation of the sample.

The strategy chosen to increase acquisition speed was to split the laser beam into a grid of beamlets focused in parallel portions of the sample via a DOE positioned along the laser pathway. If compared with other multispot microscopes in which, for example, the beam is split using a microlens array,⁷ the DOE insertion guarantees a homogeneous distribution of the light intensity over the sample, thus reducing the need for off-line processing of the images.¹⁴

This detection scheme has the advantage with respect to a CCD that optical cross-talk between the different fluorescence channels is strongly reduced.²³ This is essential when deep imaging is performed and cross-talk artifacts may completely destroy the image quality.²⁰ For this reason, in order to reconstruct a single output image with a 16 PMT microscope, it is crucial that the PMTs are equalized to the output intensity for identical input brightness. The PMTs of the detection system have been optimized for measurements of calcium dynamics in thick tissue samples and then used for the first time for Ca²⁺ imaging of acute cardiac slices. The choice of a detection scheme exploiting parallel detection performed by an array of PMTs was essential to allow deep imaging, avoiding cross-talk artifacts. However, each PMT is an independent component having different gain efficacies. To compensate for the intrinsic differences of individual PMTs, all the tubes can be equalized by independently adjusting gain and offset. The signal level can be equalized within 1%, thus compensating any source of nonuniformity in the excitation/detection path. Notwithstanding these adjustments, perfect equalization of the PMTs could not be obtained by us and others, likely due to the inherent differences in the noise level characteristic of each PMT.

Here, we showed that the multispot scanning system applied to a multiphoton microscope greatly improves its temporal resolution without worsening the spatial resolution.

First, we characterized microscope resolution by calculating radial and axial resolutions in a thick tissue phantom. This allowed us to verify that the laser could penetrate deep enough inside the cardiac tissue without losing the contrast; this is important for our study because we need to enter inside the sample for at least 50 μm (around two to three cells) to overcome the surface directly damaged by the cutting procedure. It has to be considered that all the Ca²⁺-imaging experiments were performed 150 μm below the slice surface, where we also measured the PSF, in order to overcome the cell layers suffering from secondary dysfunctions.

Once assessed, the microscope resolution of the experimental model of acute ventricular slices of murine heart has been implemented and optimized for Ca²⁺ imaging measurements in wide myocardial areas.

The feasibility of ventricular heart slices has already been reported;^{18,19} nonetheless, this model has been previously used to monitor electrophysiological parameters and as a useful model for pharmacological drug testing,²⁴ but much less for Ca²⁺ imaging. Acute myocardial slices are a good model for

fluorescence Ca²⁺ imaging because they associate the ease of the bidimensional exposition of cells under the objective with the intrinsic complex network of a 3-D tissue. A limitation in the use of murine heart slices for prolonged physiological measurements may relate to oxygen diffusion in the tissue. To reduce the impact of such a problem, we have perfused the tissue slice on both sides and used short stimulation protocols at room temperature, as suggested by Barclay with respect to a similar cardiac tissue preparation.²⁵

In this work, we showed that an MMM can be exploited not only for morphological studies in thick tissues as previously reported,²³ but also to study secondary messengers in tissue preparations or whole organs.

As we showed, the possibility to acquire full images at 16 Hz without worsening the spatial resolution opens new possibilities for studies of Ca²⁺ dynamics in intact tissues including the heart. Specifically, in cardiomyocytes physiology, releases of Ca²⁺ from the SR, Ca²⁺ sparks, are fundamental in the excitation–contraction coupling.²² Moreover, abnormal Ca²⁺ releases from the SR have been associated with different channelopathies,^{26,27} and the possibility of studying subcellular Ca²⁺ events in the intact tissue is now possible with a full-frame approach allowing the detection of macrosparks, Ca²⁺ waves, and rotors.

The use of TPE in cardiovascular research is increasing, and the need of methodological improvement is arising.^{21,28} The properties of MMM will enable the investigation of fast occurring Ca²⁺-dependent signals in living cells within intact tissues, thus expanding the methodological toolkit for fluorescence imaging which, in the last 20 years, has become an experimental technique increasingly used in biomedical research.²⁹

Acknowledgments

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- Giulia Borile** received her MSc degree in physics at the University of Padova, where she is completing her PhD in biosciences and biotechnologies, working in the laboratory of Dr. Marco Mongillo at the Venetian Institute of Molecular Medicine. This manuscript is part of her PhD project that involves the use of biophysical approaches to study cardiac arrhythmogenesis with a particular interest in microscopy and Ca²⁺ imaging.
- Biographies of the other authors are not available.