Multispot multiphoton Ca\textsuperscript{2+} imaging
in acute myocardial slices

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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 multislot 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. Multislot multiphoton microscope (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 \textit{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.

Keywords: multiphoton microscopy; multislot microscopy; calcium imaging; heart slices.

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 [Ca\(^{2+}\)] within the cells in their physiological 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 \times 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. Different solutions have been used\(^{7}\) 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.\(^{8}\) 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.\(^{4,5}\) 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,\(^{6}\) 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 \textit{in vivo}.\(^{7}\) Other techniques exploiting acousto-optic deflectors instead of galvanometric mirrors have been applied to Ca\(^{2+}\) imaging\(^{7}\) 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
plane illumination microscopy. This technique has been used for
developmental studies and is fully characterized for highly
scattering samples.

To increase the acquisition rate of TPEM, we used a parallel
scanning system based on the insertion of a diffractive optical
element (DOE) along the incident laser path, splitting the beam
into a matrix of identical beamlets with a well-defined spa-
tial 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-filed
sample image. The multiphoton multiphoton microscopy system
described in this work (MCube, developed by light4tech) 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. Sub-
sequently, the MCube was used for Ca²⁺ imaging experi-
ments 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
platform for multiphoton microscopy as it combines the pre-
servation of the multicellular myocardial structure with the
quasi-bidimensional morphology of the slice.

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 sys-
tem. The radiance 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 500 LP DC dichroic
mirror and an HQ535/50 emission filter (Chroma Technology
Corp.), was used for the detection of fluorescence emission sig-
als. 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. [3]. 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). Atrial were excised by a cut on the transversal
plane, also creating a basis surface for fixing the ventricular por-
tion of the heart to the slicer support with a commercial cyanoa-
crylate glue. Hearts from young mice were embedded in 4%
cold 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-m M 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.

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

2.3 Imaging and Stimulation

Viable heart slices loaded with Fluo-4-AM 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); sli-
ces 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 cells but, to the best of our knowl-
edge, MMM has never been employed to image cellular Ca²⁺
within 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 homo-
geneous 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(b)]. 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 configu-
ration, 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 per-
cent due to the DOE construction, Fig. 1(b)], thus providing uni-
form illumination of the entire scanned area. 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 galvonometric 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,
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 elements 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 $464 \pm 10$ 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...
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

![Fig. 2](image.png)

**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.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>FWHM radial (μm)</th>
<th>FWHM z-axial (μm)</th>
</tr>
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<tbody>
<tr>
<td>MP2100</td>
<td>2.24 ± 0.14</td>
<td>21 ± 1.2</td>
</tr>
<tr>
<td>MCube (with DOE)</td>
<td>1.30 ± 0.05</td>
<td>17.7 ± 0.7</td>
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shown in Fig. 2(a) and the characteristics of fluorescence profiles are reported in Table I.

The results obtained with the MMM were compared with that of a commercial standard, the BIORAD MP2100 multi-photon 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, 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 multi-spot 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](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics)

**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 or 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) signals from the subcellular to the multicellular range.

We used the MMM system to perform two different sets of experiments. In the first, Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) transients’ decay with a single 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. In the subsequent set of experiments, MMM was used to investigate Ca\(^{2+}\) 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 Ca\(^{2+}\) dynamics in a wide region of the slice. The elementary Ca\(^{2+}\) signals regulating the excitation–contraction coupling in heart cells are represented by Ca\(^{2+}\) “sparks,” i.e., transient and localized releases of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) through the ryanodine receptor Ca\(^{2+}\) channels.

A pace-stop protocol was used to increase the frequency of Ca\(^{2+}\) release events. Intracellular and local Ca\(^{2+}\) release events, both in the form of secondary “macroparks” [Fig. 3(b)] of approximately 4 to 6 μm in diameter, and self-sustained Ca\(^{2+}\) waves traveling through the cell [Fig. 3(c)] could be imaged simultaneously in numerous cardiomyocytes in the slice. Such cell-wide Ca\(^{2+}\) waves frequently originated from the same Ca\(^{2+}\) release hotspot and, in some cases, were underlined by organized intracellular Ca\(^{2+}\) rotors (see Fig. 4). Moreover, it was possible to appreciate that the neighboring cells displayed Ca\(^{2+}\) waves in a pattern of interdependency (Fig. 5).

### 4 Discussion

The simultaneous analysis of intracellular Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) variations in multiple cardiomyocytes.
3. A system capable of temporal resolution compatible with the dynamics to be investigated.

In standard laser scanning confocal microscopes and TPEM, 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 galvano-metric 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 beamslets 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, 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.

This detection scheme has the advantage with respect to a CCD that optical cross-talk between the different fluorescence channels is strongly reduced. This is essential when deep imaging is performed and cross-talk artifacts may completely destroy the image quality. 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 Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+}-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 Ca\textsuperscript{2+} imaging measurements in wide myocardial areas.

The feasibility of ventricular heart slices has already been reported, nonetheless, this model has been previously used to monitor electrophysiological parameters and as a useful model for pharmacological drug testing but much less for Ca\textsuperscript{2+} imaging. Acute myocardial slices are a good model for fluorescence Ca\textsuperscript{2+} 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.

In this work, we showed that an MMM can be exploited not only for morphological studies in thick tissues as previously reported 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 Ca\textsuperscript{2+} dynamics in intact tissues including the heart. Specifically, in cardiomyocytes physiology, releases of Ca\textsuperscript{2+} from the SR, Ca\textsuperscript{2+} sparks, are fundamental in the excitation–contraction coupling. Moreover, abnormal Ca\textsuperscript{2+} releases from the SR have been associated with different channelopathies and the possibility of studying subcellular Ca\textsuperscript{2+} events in the intact tissue is now possible with a full-frame approach allowing the detection of mac processoys, Ca\textsuperscript{2+} waves, and rotors.

The use of TPE in cardiovascular research is increasing, and the need of methodological improvement is arising. The properties of MMM will enable the investigation of fast occurring Ca\textsuperscript{2+}-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.

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References


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.