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The BrightEyes-TTM: an open-source time-tagging module for fluorescence lifetime imaging microscopy applications

by

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I would like to dedicate this thesis to my family and my loving wife.

Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements. This dissertation contains fewer than 65,000 words including appendices, bibliography, footnotes, tables and equations and has fewer than 150 figures.

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Abstract

The aim of this Ph.D. work is to reason and show how an open-source multi-channel and standalone time-tagging device was developed, validated and used in combination with a new generation of single-photon array detectors to pursue super-resolved time-resolved fluorescence lifetime imaging measurements.

Within the compound of time-resolved fluorescence laser scanning microscopy (LSM) techniques, fluorescence lifetime imaging microscopy (FLIM) plays a relevant role in the life-sciences field, thanks to its ability of detecting functional changes within the cellular micro-environment. The recent advancements in photon detection technologies, such as the introduction of asynchronous read-out single-photon avalanche diode (SPAD) array detectors, allow to image a fluorescent sample with spatial resolution below the diffraction limit, at the same time, yield the possibility of accessing the single-photon information content allowing for time-resolved FLIM measurements. Thus, super-resolved FLIM experiments can be accomplished using SPAD array detectors in combination with pulsed laser sources and special data acquisition systems (DAQs), capable of handling a multiplicity of inputs and dealing with the single-photons readouts generated by SPAD array detectors.

Nowadays, the commercial market lacks a true standalone, multi-channel, single-board, time-tagging and affordable DAQ device specifically designed for super-resolved FLIM experiments. Moreover, in the scientific community, no-efforts have been placed yet in building a device that can compensate such absence. That is why, within this Ph.D. project, an open-source and low-cost device, the so-called BrightEyes-TTM (time tagging module), was developed and validated both for fluorescence lifetime and time-resolved measurements in general.

The BrightEyes-TTM belongs to a niche of DAQ devices called time-to-digital converters (TDCs). The field-gate programmable array (FPGA) technology was chosen for implementing the BrightEyes-TTM thanks to its reprogrammability and low cost features. The literature reports several different FPGA-based TDC architectures. Particularly, the differential delay-line TDC architecture turned out to be the most suitable for this Ph.D. project as it offers an optimal trade-off between temporal precision, temporal range, temporal

resolution, dead-time, linearity, and FPGA resources, which are all crucial characteristics for a TDC device. The goal of the project of pursuing a cost-effective and further-upgradable open-source time-tagging device was achieved as the BrigthEyes-TTM was developed and assembled using low-cost commercially available electronic development kits, thus allowing for the architecture to be easily reproduced. BrightEyes-TTM was deployed on a FPGA development board which was equipped with a USB 3.0 chip for communicating with a host-processing unit and a multi-input/output custom-built interface card for interconnecting the TTM with the outside world. Licence-free softwares were used for acquiring, reconstructing and analyzing the BrightEyes-TTM time-resolved data.

In order to characterize the BrightEyes-TTM performances and, at the same time, validate the developed multi-channel TDC architecture, the TTM was firstly tested on a bench and then integrated into a fluorescent LSM system. Yielding a 30 ps single-shot precision and linearity performances that allows to be employed for actual FLIM measurements, the BrightEyes-TTM, which also proved to acquire data from many channels in parallel, was ultimately used with a SPAD array detector to perform fluorescence imaging and spectroscopy on biological systems.

As output of the Ph.D. work, the BrightEyes-TTM was released on GitHub as a fully open-source project with two aims. The principal aim is to give to any microscopy and life science laboratory the possibility to implement and further develop single-photon-based time-resolved microscopy techniques. The second aim is to trigger the interest of the microscopy community, and establish the BrigthEyes-TTM as a new standard for single-photon FLSM and FLIM experiments.

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Chapter 1

Introduction

In this introductory Chapter, I will rst explain the truly motivations at the basis of this Ph.D. work, then I will provide to the readers the basic microscopy notions requested to understand the real and speci c aims of this Ph.D. work.

1.1 Motivations

Fluorescence laser scanning microscopy (FLSM) is a powerful imaging tool for investigating both function and structure of biological systems (1). Indeed, FLSM not only provides sub-cellular spatial resolution, live-cell harmony, and molecular speci city and sensitivity, which are general properties of uorescence microscopy, but also compatibility with many advanced microscopy techniques. For example, confocal and uorescence lifetime microscopy, which are two of the most important FLSM synergistic combinations and which represent two topics of this Ph.D. work. Confocal microscopy provides optical sectioning, thus allowing three-dimensional imaging also in relatively thick samples. Fluorescence lifetime microscopy provides the possibility to investigate the functions of different sub-cellular structures and/or to understand their chemical environments.

However, current FLSM implementations suffer from a fundamental limitation: the way how they build an image inevitably lose a series of information encoded in the uorescence signal. In the following, I will describe how the sample's image is typically formed in a uorescent laser scanning microscope, and I will explain why this image formation process loses important sample information. In a conventional FLSM experiment, an objective lens focuses an excitation laser beam at a speci c position in the sample plane, known as probing (or detection) region. Then, the same objective lens subsequently collects the emitted uorescence and, together with a tube lens, projects the light onto the sensitive area of a

detector, which is generally constituted by a single element sensor, such as photo-multiplier tube (PMT). Indeed, while the microscope is able to produce an image of the probing region, the sensor integrates all the uorescence light regardless its spatial distribution: in short, from a three-dimensional light intensity distribution $I(x^0, y^0, t)$, the detector generates only a temporal distribution $I(t)$. Successively, the signal is conventionally connected to a data-acquisition (DAQ) system, whose aim is to integrate the light intensity signal collected by the detector along the whole pixel-dwell time, i.e., the time that the microscope maintain stationary the probing region in a speci c sample position (pixel). In short, from a temporal light intensity distribution $I(t)$, the DAQ generates a single intensity value I associated to a speci c sample position (pixel). The probing region is usually raster-scanned across the sample with the use of a dedicated laser beam scanning device to form the nal bi-dimensional (or three-dimensional) digital image. It is clear that, while the optical microscope is able to provide a spatiotemporal light intensity distribution for each speci c position of the probing region across the sample, the way how the image is recorded completely cancels out all this information, producing only a single intensity value for probing region (pixel).

The recent introduction of new single-photon detector, such as the asynchronous read-out single-photon avalanche diode (SPAD) bi-dimensional array detector, rst developed by our group (2), potentially allows for completely overcoming this limitation. Contrarily to a conventional single-element detector, a SPAD array is constitute of multiple element sensors distributed to sample a bi-dimension surface. Furthermore, each element is capable of giving as a output a single-photon readouts with a precision of few tens of picoseconds. These properties allow for imaging of the probing region of a uorescence laser scanning microscope with, in principle, unlimited temporal resolution. In other words, this novel class of detector potentially give access to the spatiotemporal light intensity distribution $I(x^0, y^0, t)$ for each probing region in the sample. As example of the perspectives open by this novel detector is super-resolution uorescence lifetime imaging. If a SPAD array detector is used in conjunction with (i) a pulsed laser source to excite the sample, and (ii) a special DAQ to acquire the SPAD array outputs in synchronization with the excitation events (i.e., the delay between the excitation pulse and the photon registration, the so called photon-arrival time), it is possible to implement sub-nanosecond time-resolved measurements. While the images of the single probing regions allows to reconstructed a super-resolution image of the sample, the sub-nanosecond time resolved measurement allows to assign to each pixel of the super-resolved image a uorescence lifetime value. Another interesting perspectives are the combination of uorescence uctuation spectroscopy with uorescence lifetime analysis: a

technique able to correlate the dynamics properties of a bio-molecule with information about its chemical environment or its structural changes.

However, all these perspectives risk being wasted if SPAD arrays detector are not combined with adequate and specialized DAQ systems, capable of time-resolving, harvesting and handling the high throughput of information generated by the single-photon detector arrays. From the point of view of the DAQ system, SPAD array detectors are made up by multiple element sensors of which the individual output needs to be acquired thus the DAQ system would require many input channels working in parallel. Although different commercial time-tagging devices are already present on the market, they can achieve multi-channel (> 20 input channels) capabilities only when inter-connecting several bulky electronic modules. The instrumentation market precisely lacks a true stand-alone single module DAQ that can all-together handle a multiplicity of input channel. Moreover, the commercially available time-resolved apparatuses implement general-purpose time-tagging functions which are not speci cally conceived for uorescence microscopy applications. In addition, these off-the-shelf instrument solutions focus their performances in yielding time resolutions as low as 1 ps which is admirable but out of the scope of uorescence lifetime measurements in which a resolution of 100 ps would still perfectly work.

All-in-one solutions, with the DAQ system being integrated into the detector's body, were also developed[3, 4]. Although being very compact, such type of solutions, lack exibility in the sense that if, due to the constant technological progress, a new detector is developed also a new DAQ system will have to be physically re-engineered, to match the new detector's specs. Another important aspect to take into consideration is the fact that time-tagging DAQ devices belong to a niche of evolved/cutting-edge instrumentation and do not have the same price ranges of mass-consumed electronics, this aspect may inhibit the adoption and the widespread of time-tagging devices in FLSM limiting the potentiality of single-photon-based applications and techniques.

Indeed, if, on one hand, lot of investments and developing efforts are placed in ne-tuning the SPAD technology and more in general multi-element single-photon detector solutions, on the other, the lack of a user-customized and open-source DAQ system is felt in the microscopy and spectroscopy community.

In order to ll this gap and, at the same time, overcome the disadvantages/limitations of current DAQ system technology, within this 3-years-long PhD project an open-source multi-channel timetagging DAQ module (TTM) based on a eld-programmable gate array (FPGA), that can temporally tag single-photon events as well as synchronisation events was engineered and developed. TTM's strength resides in being an open-source single-board

device that can handle multiple channels in parallel working in a stand-alone modality. The TTM is conceived to work as a passive plug-n-play device that can be connected to any type of detector and uorescence microscopy setup with eventually minimal hardware modi cations. The open-source feature confers TTM the primacy of being a uorescence microscopy bespoke as well as a cost-effective solution that can be easily built and further upgraded, modi ed, and customized by the microscopy-makers.

I believe this TTM-DAQ module can unlock the real potential carried along with single-photon information and open up new research lines within FLSM-FLIM applications. TTM-DAQ project will give the possibility of pursuing state-of-the-art time-tagging measurement experiments in many labs and hopefully also establish, with no precedents, an open-source benchmark for time-resolved instrumentation.

1.2 Fluorescence laser scanning microscopy

Apart from its intrinsic morphology and spatial organization, the main and most measured properties of a sample are: optical re ectivity (5), light absorption and emission spectrum (6), light polarization (7) and uorescence emission (8; 9). Amongst these, notably uorescence plays a more prominent role. Firstly observed by Herschel in 1845 (10) and subsequently studied by Stokes, who coined the term uorescence in 1852 (11), (uorescence is the emission of light by a molecule that has absorbed light as consequence of an external excitation process.The detection of uorescence emission is widely used in the life sciences research world for both structural and functional analysis of biological material.

In FLSM, an object or a sample is scanned point by point by a focused laser beam. In this way, a spatial sampling of the object is performed and the nal microscope image is reconstructed in a post-processing phase. The main components of a conventional FLSM setup are (i) a laser illumination source (ii) a scanning system and a (iii) light/photons detector. In a FLSM apparatus, the laser (i.e. the illumination source) is focused to a spot size meanwhile the scanning system allows for raster-scanning the laser light onto the sample and ultimately, the detector collects the back-scattered uorescence light from the sample. In one of the most basic FLSM setup a single laser source is needed in combination with a single-element detector where the uorescence light from each scanned location of the sample is registered by the the detector. Usually, optics component (lenses, mirrors and optical lters) are integrated in a FLSM setup in order to achieve higher object magni cation, depth of focus and eld of view (FOV) (12; 13; 14).

Worth mentioning is the fact that traditional FLSM came of age when new uorescence laser scanning imaging systems have been developed to overcome some limitations connected with the FLSM techniques (15). For example, increased magni cation and numerical aperture (NA) optics have been developed and employed for spatially-resolving the sample features in a more accurate way (16). And, most importantly, the use of a pinhole was also introduced in order to reject out-of-focus light, in respect to the imaging scanning plane, coming from the sample (CLSM) yielding images with higher contrast and resolution (17).

1.3 Fluorescence confocal laser scanning microscopy

In conventional FLSM setup the reconstructed image of the sample is affected by the blurring contribution coming from out-of-focus regions which reduce the contrast of the in-focus light broadening the system point-spread-function (PSF) and ultimately affecting the nal image resolution (18). CLSM overcomes this problem by introducing a confocal aperture (such as a pinhole) in the emission beam path to reject the out of focus contribution (19) while also reducing the full-width at half-maximum (FWHM) PSF pro le. Similarly to FLSM, in CLSM a point-like laser source is focused by an objective onto a sample. The spatial extension of the focus spot on the sample is determined by the wavelength (λ), the NA of the lens, and the quality of the image formation. Like in FLSM, the image spot is usually focused through the same lens onto a pinhole aperture and ultimately onto a single-element detector. The pinhole aperture is situated at a conjugate focal plane. Fundamentally, the principle used in a CLSM microscope, thanks to the introduction of the confocal aperture, allows to block the light coming from object planes above or below the image focus plane. Consequently, all out-of-focus optical background is removed from the image and the confocal image is an “optical section” of the sample (20).

CLSM turned out to be an indispensable technique for biomedical research being noninvasive, nondestructive and allowing for optical-sectioning of the sample. Furthermore, thanks to the improvements made in arti cial uorescence labelling technology (i.e. the capability of selectively attaching an arti cially synthesized uorophore to a speci c biological molecule or structure) CLSM helped to solve a plethora of various and different biological problems (10; 21; 22; 23; 24; 25).

Spatial resolution is another important advantage of CLSM, as shrinking the optical pinhole aperture makes it possible to further reduce the spatial extension of the CLSM PSF by up to a factor of $\sim \sqrt{2}$ (26; 27) with respect to conventional diffraction-limited microscopy thus improving the system lateral-resolution (28). However, the reduction of the PSF does

not always lead to a resolution enhancement, because reducing the pinhole diameter also causes a heavy signal loss, and thus a decrease of the overall signal-to-noise ratio (SNR) (29).

New FLSM-based techniques, in which a squared-shape multi-element detector sensor was used, implemented a laser scanning methodology known as image scanning microscopy (ISM) (30) which overcame the SNR limitations of CLSM effectively obtaining the theoretically $\sqrt{2}$-fold resolution improvement predicted for CLSM without implying the use of a pinhole aperture. Yet, more recently, a new ISM approach based on a SPAD array detector was introduced (31).

1.4 Fluorescence image scanning microscopy

In a nutshell, ISM is a super-resolution microscopy technique that allows to overcome the diffraction barrier by a factor of $\sqrt{2}$ without reducing the overall image SNR. In ISM the single-element photo-detector is replaced by an imaging detector and the pinhole is removed so that each sub-element of the detector effectively acts as a virtual pinhole. This allows all the light to reach the image plane to be collected with a sub-Nyquist sampling (32). Similarly to CLSM, ISM requires an acquisition of the image of the excitation and detection region for each scanning position of the sample. The final ISM image is then obtained by computationally combining the information contained from all the acquired images i.e., after each scanning the microscope produces one confocal image for every element (pixel) of the detector array (33). As said, ISM cannot be accomplished using a single-element detector like photomultiplier tubes (PMTs) (34) or microchannel plates (MCPs) (35), which, so far, despite limitations like fragility, intrinsic deterioration with usage, high cost, bulkiness, and operation complexity, covered a major role in the field of FLSM and CLSM mainly because two advantages (i) large detection area and (ii) high measurement dynamic and spectral range (36; 37; 38).

Until today, ISM system configuration have been mainly implemented using conventional cameras, trading off a low imaging speed due to the limited camera frame rate (30; 39). Recently, the imaging speed limit was addressed by the Airyscan implementation, for which a 2D bundle of optical fibers is coupled to a linear array of GaAsP PMTs (40). However, this solution needs intercalated expensive and bulky electronics for sampling the fluorescence and shows some of the distinctive drawbacks of vacuum-based detectors like fragility and high cost.

De novo, a new kind of photo-detectors, known as SPAD detectors, are gaining ground in ISM applications thanks to their reliability, robustness, ease of operation, high detection ef ciency, low timing jitter, and their integratability with read-out circuits, allowing for the development of multi-element arrays (2). The real advantage of SPAD sensors is their compatibility with microelectronic circuits, which allows the integration of dedicated electronics into the same silicon chip for creating mono- as well as bi-dimensional arrays of detectors which practically rules out the need for extra electronics. Moreover, their higher temporal resolution that allows for effectively implementing single-photon imagers with photon-timing capability (41).

However, both for CLSM and ISM, data derived from uorescence intensity, can be affected by variations in laser excitation intensity, by uorescent dye concentrations, by photobleaching (i.e. photochemical alteration of a uorophore molecule such as it is permanently or transiently unable to uoresce) and instrumental drift effects (focusing issues, detectors gain response, laser misalignment) (42). It is therefore dif cult performing quantitative measurements relying only on the uorescence intensity. Furthermore, it is nearly impossible to distinguish different uorescent probes with very similar uorescence spectra (43). In contrast to the limitations of uorescence intensity another property of the emitted light that can be measured and from which it is possible to derive quantitative biochemical parameters of live cells and tissues, such as oxygen content (44; 45), pH (46; 47), metabolic state (48), viscosity (49), ion concentrations (50), temperature (51) is uorescence lifetime.

Contrarily to the Airyscan approach, which still holds back the temporal information related to photon arrivals thus preventing the implementation of uoreescence lifetime imaging microscopy (FLIM) (or similar techniques), SPAD array detectors, with single-photon (SP) asynchronous detection, and picosecond time-resolution ability and fully independent elements readout, are a very effective solution to overcome all the above mentioned limitations, and to implement newly ISM as well as FLIM methodologies.

1.5 Fluorescence lifetime imaging microscopy

Conventionally, uorescence lifetime ($\tau(\lambda)$) is de ned as the average time that a uorophore, after being excited, remains in its excited state By measuring the uorescence lifetime at different position in the sample (as per FLSM techniques) it is possible to build up images re ecting the spatial variations of the uorescence lifetime. This method is called uorescence lifetime imaging microscopy (FLIM) (52; 53). Unlike uorescence intensity-based measurements, FLIM provides unique advantages as for example it is independent

of uorophore concentration. FLIM measurements are also immune to light scattering effects that can potentially modify the detected uorescence intensity. Moreover, FLIM is a self-referenced measurement (i.e., independent of absolute detected intensity), thus FLIM experiments do not require the throughput calibration steps that are needed for intensity based experiments. In addition, uorescence lifetime is an absolute measurement that can be repeated across numerous device con gurations after accounting for the instrument response function (IRF) of that device. Thus, artifacts caused by nonuniform illumination, which would greatly affect intensity measurements, are mitigated by measuring the lifetime.

For these reasons FLIM gained popularity in the bio-scienti c community, thanks also to its high sensitivity to changes in molecular environment and molecular conformation of the sample (54; 55; 56). It has been used in auto uorescence molecular imaging (i.e. exploiting the uorescence response of endogenous uorophores) the to study cellular metabolism and is gaining momentum being used as a contrast mechanism to image live tissues allowing for uorescence-guided surgery (57; 58). As well as with endogenous uorophores, FLIM is also used in conjunction with exogenous uorescent molecules that are for example capable of monitoring parameters (e.g temperature, ion concentrations, pH etc. etc.). Relying both on exogenous and endogenous uorophores, FLIM is suitable for monitoring and direct measuring numerous biochemical processes in cells and tissues including also disease progression, drug delivery and vaccines ef cacy (59; 60; 61).

Traditional continuous wave laser (CW) sources are not suitable for accomplishing FLIM measurements as continuously illuminating the sample does not allow for discriminating the difference in the change of state (excited vs. ground state) of the uorophore over time Therefore, in a FLIM setup, optics and scanning components, have to be coupled with (i) pulsed laser sources (PW) for illuminating the sample and (ii) new types of light detection technologies such as avalanche-photodiode (APD) (62), SPAD (63), hybrid detectors (HyD) (64) and superconducting nano-wire detectors (65) (which allow for individual (single) photons to be detected. Precisely for this reason, the SPAD array detector, thanks to its single-photons asynchronous readout capabilities, was used, in conjunction with a PW laser, for FLIM measurements, namely implementing super-resolution uorescence lifetime image scanning microscopy (FLISM) technique (66).

1.5.1 Measuring uorescence lifetime

With FLIM techniques it is possible to measure the uorescence decay rate of a uorophore which falls in the timescale of few picoseconds to hundred of nanoseconds. In order to

accomplish the measurement of such short time intervals LSM setups have to be coupled with PW laser sources, fast response SP detectors and fast DAQ cards able to assess and register the time of arrival of single photons. Talking about fast DAQ electronics card, FLIM measurements can be carried out using a time-domain (TD) (67; 68; 69) or a frequency-domain (FD) (70; 71; 72) (evolved thanks to (73) into digital-frequency-domain (DFD)) approach. State-of-the-art TD uorescence lifetime measurements use a short laser pulse for exciting the uorophore and then record, using time-resolved DAQ electronics, photon arrival times (time of ight - ToF) in respect to the excitation pulse. By sampling and collecting several photons together with their arrival time (time-tag), it is possible to build a photon arrival times histogram, known as time-correlated-single-photon-counting (TCSPC) histogram (74) (Fig. 1.1 b). TCSPC histogram is substantially a plot of the uorescence decay curve (rate) over time t_{ij} can ultimately be calculated from the TCSPC histogram using either a tting procedure (75) or the FLIM-phasor approach (76; 77). In a FLSM-FLIM system the TCSPC histogram is reconstructed as a function of the spatial coordinates (x, y) describing an image (Fig. 1.1 b). The nal result of a FLIM measurement is an image in which the pixels are color-coded according to the bi-dimensional colormap that encodes uorescence lifetime values as shown in Fig. 1.1 c.

On the other hand, DFD uses the same TD measuring setup (short pulses PW laser source and fast SP detectors) but, for DFD, single photons ToF is calculated using an heterodyne principle. In DFD electronics, a digital frequency (known as sampling frequency) that beats with the PW laser repetition frequency rate is generated and used to make a a digital counter (window counter) advance thus creating sliding time windows within the PW laser repetition period. As soon as a photon is detected, the value of the window counter is registered and used as ToF. Similarly to TD, in order to reconstruct the uorescence decay curve, photons ToFs are used to build the TCSPC histogram (78; 79).

The TD-TCSPC approach is commonly used in LSM-FLIM biological applications as it combines high time resolution and high photon ef ciency. In a TD-TCSPC-based FLIM architecture the sample is repeatedly scanned by an high repetition rate (up to 80MHz) PW laser beam, and a detector (or more than one) detects single photons of the uorescence emission signal returning from the sample. Each detected photons is characterized by its time-tag (i.e. its time of arrival within the laser pulse period) and the coordinates of the laser spot in the scanning area in the moment of its detection. The image recording process creates a distribution of photon ToFs (i.e. the TCSPC histogram) for each of the scanned position: the acquisition results into a 3D image having pixels for the rst two dimensions

Figure 1.1 TCSPC histogram and FLIM image. a Principle behind photon arrival timing for TCSPC histogram reconstruction. After exciting the sample with a pulsed laser source and systematically registering the time of arrival of many uorescence (single-)photons, a histogram, that represents the uorescence decay is built. b Basic principle of FLIM, where a uorescence decay TCSPC histogram is reconstructed for every pixel of the image (x-y) leading to a 3D image $(x; y; Dt)$, where $(x; y)$ are the spatial coordinates of the beam scanning system, and Dt is the dimension of the TCSPC histogram in each pixel $(x; y)$. example of a FLIM image: to each pixel corresponds a lifetime value which is color-coded according to the bi-dimensional colormap (top) and shown in the FLIM image (bottom). Scale bar 2.5 mm.

and containing TCSPC histograms for each pixels along the third dimension over a large number of time channels (80) (Fig. 1.1 b).

1.5.2 DFD-FLIM acquisition systems

The DFD approach is traditionally implemented in a FPGA chip. Within the FPGA, an electronic circuit is synthesized and used to sample and tag uorescence single-photons. The DFD-FPGA circuit implements a heterodyne sampling architecture in which digital square sampling windows are used to time-gate the incoming photons and register their arrival times. The DFD methodology creates a low frequency replica of a uorescence decay exploiting the digital time-gating heterodyne principle in combination with the periodicity of the PW laser source. The slow frequency replica is generated by a convolution between the uorescence single-photon signals and the square digital sampling windows. Thanks to the heterodyne

principle the time-gating (photons sampling) can be accomplish with ~48 ps precision yielding phase-sampling performances comparable to a TD-TCSPC device. If on one hand DFD-FLIM systems are capable of real-time FLIM-phasor analysis and have high-precision phase measurement features, on the other they lack, so far, high temporal resolution (~15 ns (79)). The temporal resolution of the acquisition electronics impacts the overall instrument response function (IRF), the lower the temporal resolution the broader the IRF would be. Although a broad IRF is not a crucial parameter for uorescence lifetime analysis as long as its shape and characteristics are known a priori - as explained in (81) -, TD-TCSPC devices are mostly preferred over DFD ones when dealing with FLIM measurements.

1.5.3 TD-TCSPC FLIM acquisition systems

The core of TCSPC devices is the electronics for the conversion of arrival times of photons into a digital data format. Commonly used TCSPC-FLIM devices implement either a time-to-amplitude-converter-analog-to-digital-converter (TAC-ADC) principle or a time-to-digital converter (TDC) principle (82). The TAC-ADC principle converts the ToF of each single-photon into the amplitude of an analog (voltage) signal and then into a digital data record (83; 84). TAC-ADC circuits are usually developed using application-speci c integrated circuit (ASIC) technology. ASIC-based TAC-ADC deliver extremely high time-resolution (< 0.5 ps) and precision (~1 ps). Contrarily to TAC-ADC circuits, the TDC principle it is an all-digital measurement methodology that uses the delay in a chain/line of logic gates as a timing reference for assessing ToF time. The delay chain approach delivers a time resolution down to a few ps yielding measurement precision of tens of picoseconds (85). If (TAC-ADC systems can only be implemented on ASICs, TDCs can actually be implemented on special electronics known as eld-programmable-gate-array (FPGA) (86).

1.6 Aim of the work

In order to pursue time-resolved uorescence lifetime-based measurements (and applications) making the most out of SPAD array detectors usage characteristics and performances (multi-element single-photon asynchronous readouts), in this PhD thesis work, I propose an open-source multi-channel time-resolved and standalone DAQ platform. This special DAQ module, named BrightEyes-TTM, is speci cally designed to work as TD-TCSPC device implementing a delay chain-based digital TDC approach.

The BrightEyes-TTM can perform multiple photon- and reference-channels time-resolved samplings in order to implement current and future single-photon-LSM/FLIM techniques. The BrightEyes-TTM platform is based on a commercially available and low cost evaluation board, equipped with a state-of-the-art FPGA and a series of I/Os connectors. I/Os connections grant an easy interface of the BrightEyes-TTM board with a FLSM environment, the SPAD array detector and an host processing unit. The FPGA technology was chosen to grant both quick prototyping and versatility, and to meet the future requests from new SP-LSM applications. The current implementation was conceived and designed having in mind the uorescence applications and the SPAD array detector characteristics, providing a good compromise between cost, photon-timing precision and resolution, temporal range, electronics dead-time and maximum photon- ux. Indeed, BrightEyes-TTM was engineered to operate as a single-board passive standalone platform for two main reasons (i) if in the future new type of the detectors will reach the market the BrightEyes-TTM can still be used, due to its versatility, by simple hooking some connection cables to them (ii) other commercially available time-tagging platforms, in order to achieve multi-channel capabilities, are made up by several bulky modules that need to be interconnected together impacting the ability of such systems to be easily accommodated and integrated in a lab environment. Additionally, the low FPGA resource needed by the current implementation ensures the highly scalability of the architecture, potentially enabling more channels and, in general, new applications.

Chapter 2

FPGA-based time-tagging architectures

The field programmable gate arrays (FPGAs) are semiconductor devices that are based around a matrix of configurable logic blocks (CLBs) connected via re-programmable interconnections. FPGAs can be reprogrammed to achieve and implement desired application or functionality requirements after manufacturing. Originally engineered for electronic devices prototyping, thanks to their low cost and reprogrammability, FPGAs are gaining active momentum and a relevant role in various industrial and research fields as primary used electronic components: fluorescence microscopy is one of those. Indeed, FPGAs are used in FLSM for handling many input channels in a parallel and for, due to the possibility of accessing low level basic silicon components (CLBs), measuring time intervals with picosecond precision by exploiting several configurable architectures known as time-to-digital converters (TDCs).

2.1 FPGAs

In recent years, the development of complex and compact high performance electronic controller systems has changed the world of very large scale integration and electronic design automation techniques (87; 88). The speed and re-programmable performances of new electronic components as well their flexibility for digital designs implementation have created new opportunities in terms of new applications and, consequently, opened up new markets. This is specially true for a specific hardware technology known as FPGAs. FPGAs combine low cost digital electronics development use of convenient software designing, programming tools and, an always growing, integration capability with external peripherals (89; 90; 91). The FPGA technology is being adopted in various fields of applications: from telecommunications (92), signal and image processing (93; 94), medical equipment (95; 96), robotics (97; 98; 99; 100) and machine learning (101; 102; 103). Yet, also in other application

elds FPGAs are revolutionizing the way digital signals are handled and processed. Indeed, focusing on LSM and FLIM applications, FPGAs have already been used with success for TD (104; 105; 106; 107) and DFD (73; 79) implementations. This is mainly because FPGA-based circuit implementation, thanks to their architecture scalability, can match current and future challenges of LSM and FLIM approaches.

FPGAs carry along many inherent advantages. First of all, low costs: FPGA design can be molded only on the speci c needs of the application to implement shortening the time-to-market. Secondly, VLSI design coupled with FPGAs, allows also for analog interface on FPGAs, thanks to the use of system-on-a-chip (SoC) (108; 109). FPGA-SoC integrates analog and digital capabilities on the same silicon chip, yielding an high application versatility (110). Lastly, thanks to FPGAs parallel architecture computation abilities, tasks execution time is drastically reduced allowing FPGA-based designs to reach speed-performances of analog devices but without their downsides: lack of exibility, as analog circuits cannot be reprogrammed, and performance drifts due to intrinsic nature of analog signals (111). FPGAs can be included in the wide family of programmable and re-programmable digital-logic components (112).

2.1.1 FPGA system architecture

An FPGA consists of a matrix of customisable logic blocks linked to each other via an interconnection network which is completely reprogrammable. The con guration of the logic blocks and the arrangement of the interconnections, which determine the actual and overall FPGA circuit functionalities, are controlled by memory cells. Several con gurable memory technologies exist, the most exible and used one is the static random access memory (SRAM)-based FPGA technology (113; 114; 115).

The generic architecture of an FPGA, shown in Fig.2.1, is composed of a mosaic of con gurable general purpose logic blocks (CLBs). CLBs can be considered as the basic brick of the FPGA. A CLB consists of a look-up table (LUT), which can be con gured either as memory element or a combinatorial mathematical function. A carry look-ahead data (CARRY chain) path is also included in a CLB. The CARRY element can be used to build ef cient arithmetic operators or it can be used to create controlled-delay paths within the FPGA fabric. Lastly, a D-type ip- op (DFF), with all its control inputs (synchronous or asynchronous set/reset, enable), allows for correctly registering the output of the logic cell (Fig. 2.2). The CLBs architecture corresponds to the fundamental unit of an FPGA, and it can be seen as a small digital control and processing unit since the registered output

Figure 2.1 FPGA architecture I/O pins are used to interface and connect the FPGA with the outer world; the intimate structure of and FPGA is constituted by a mosaic of logic blocks connected with each other via the interconnect resources routed amongst the different logic blocks using switch blocks. A switch block is a routing unit that can couple and connect the interconnect resources.

can be con gured as an input of the same CLB. Zooming out from the FPGA architecture: CLBs are surrounded by a ring of con gurable input/output pins (IOBs). Modern FPGAs can reach >1000 user IOBs thus yielding the capability of handling multiple I/Os at the same time. CLBs and IOBs communicate each other through a (re)-programmable interconnection network. The way the interconnection network is programmed i.e. the way CLBs and IOBs work together, creates a digital circuit design speci cally dedicated to accomplish tasks for a determined end-application (116; 117; 118).

Nowadays Xilinx Inc. (San Jose, CA, United States) and other FPGA manufacture companies have introduced inside this logic blocks mosaic architecture some dedicated and specialized components such as random access memories (RAM), digital signal processing (DSP) accelerators (119; 120), high-speed clock management circuitry (PLL, MCMM) (121), serial transceivers (SerDes) (122), embedded hard processor cores such as ARM (Xilinx UltraScale FPGA family devices) and soft processor cores such as the MicroBlaze (123; 124) in order to boost FPGAs performances and potentialities. The high performance, together with the lower costs and relatively small time-to-market, turns FPGA-based architectures alluring for TDC implementations within TD-TCSPC applications. FPGA-based TDC,

Figure 2.2 FPGA logic cell structure

being unconstrained by ASICs downsides, can fully exploits circuit scalability and re-programmability allowing for circuit designs to evolve and adapt over time (125; 126; 127; 128).

2.2 FPGA-based TDCs

In the last two decades, FPGA technology has demonstrated massive improvements, from a technical fabrication perspective to a strengthening and usability of development tools. This rapid growth in performances and capabilities allowed FPGA to close the gap, with ASIC circuits, transforming FPGA-based systems into a no longer prototype-only architectures (129; 130; 131). That is mainly why FPGA have been used also to measure time intervals with sub-nanosecond precision, exploiting a speci c type of design architectures known as time-to-digital-converters. In the context of FLSM and FLIM applications, FPGA-based TDCs, which recently reached time resolutions of ~10 ps (132) (which is more than precise enough when dealing with t_{fl} measurements) are getting to the same level as ASICs (133; 134; 135; 136). Moreover, the continuously-increasing number of CLBs available in FPGAs also contributed to the development of multi-channel and sophisticated TDC architectures. Indeed the literature of FPGA-based TDCs is full of different and various approaches, each one characterized by a unique circuit design implementation strategy in order to achieve ner time-resolutions and higher sampling-rates (137; 138; 139). That is also why a homogeneous TDC architecture categorization is hard to draft. Machado et al. (140) proposed a standardized taxonomy for classifying FPGA-based TDC architectures (Fig.2.3). Such taxonomy, was conceived considering the main circuit architecture involved in the overall system design.

From a literature analysis the most used and developed FPGA-based TDC con gurations are based on tapped-delay-lines (TDLs) (141; 142). In the next sections the main and most

Figure 2.3 FPGA-based TDC taxonomy. The main and most used FPGA-based TDC types are the coarse counter approach, the shiftef clock sampling technqie, the delay line method and the differential-delay line architecture.

used FPGA-based TDC architectures, shown in Fig. 2.3 are described putting more accent on TDC-TDLs systems (86; 143).

2.2.1 Coarse counter TDC

The coarse counter is the simplest FPGA-based TDC architecture that can be implemented. The core element is a digital counter that increments its value at a speed which depends on the clock frequency the counter gets supplied with (Fig. 2.4). The frequency value sets consequently the time resolution of the TDC architecture design. With the progress in FPGA development, the maximum operating frequency of a chip has increased reaching hundreds of MHz. Xilinx datasheets report a maximum working limit frequency of ~800 MHz system clock yielding time resolutions as low as ~1.25 ns. In order to achieve resolution below hundreds of picoseconds, a system clock frequency higher than 10GHz is required: at present day, unfortunately, FPGAs are still not feasible for generating such high frequencies, but, other techniques could be used to achieve < 1 ns such as the shifted-clock-sampling architecture (144; 145).

2.2.2 Shifted clock sampling TDC

Another TDC approach that can be pursued using FPGA platforms is the shifted-clock-sampling SCS-TDC (a.k.a. phase detection TDC). Similarly to the coarse counter approach, also starting from an FPGA clock frequency, a SCS-TDC architecture uses intrinsic functions of PLL (phase-locked-loop) & MCMM (mixed-mode clock manager) logic blocks to create multiple replicas of the same input clock signal but having different phase shifts (146; 147; 148; 149; 150). This mechanism ultimately creates a sub-clock-period-short time-windows allowing for sampling the input signal (i.e. the single-photon signals) with a ner resolution with respect to the period of the clock used (Fig. 2.5). Speci cally, for a SCS-TDC the time resolution t is equal to:

Figure 2.4 Coarse counter TDC. Schematic representation of a coarse counter TDC receiving as input a FPGA system clock and returning as output, a digital integer values (top); working principle of a coarse digital counter: at every rising edge of the FPGA system clock the counter increments its value over time. Given the actual FPGA technology the maximum operating frequency is around 800MHz yielding a time resolution of about ~1.25 ns (bottom).

$$t = \frac{T_{clk}}{N_{phases}} \tag{2.1}$$

where T_{clk} is the period of the clock used in the design and N_{phases} is the number of phase shifts generated by the clock-manager components. SCS-TDC architectures, apart from being of easy implementation, offer great system linearity while being able to achieve resolutions as low as hundreds of ps (151, 152, 153). The main drawback of SCS-TDCs, when compared to other architectures such as delay-line approaches, remains its limited resolution.

2.2.3 Delay line TDC

A delay-line TDC, also called flash TDC or tapped-delay-line TDL-TDC is the most popular FPGA-based TDC architecture because of its simplicity, low latency, reliability and high-temporal resolution (154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185). The core constituent of a TDL-TDC module is a tapped delay line (TDL) The TDL is made

Figure 2.5 SCS-TDC. Schematic representation of a coarse counter TDC receiving as input an # 4 shifted FPGA system clocks an returning as output sub-clock-resolved values (top); working principle of an SCS-TDC: the FPGA system clock period is sub-divided in N_{phases} = 4 time intervals (k_{1-4}) exploiting the different phases of the other input clocks (90°, 180°, 270°). Every k_i can have a time resolution as low as hundreds of ps (bottom).

up by a series of small delay elements joined in a chain architecture and is used to delay an input signal (usually called START signal i.e. the signal from which the ToF is measured) with respect to a reference-sampling signal (usually called STOP signal i.e. the signal used as a time-reference for measuring the ToF). From the delay characteristics of each delay element, it is possible to match the distance covered by the START signal along the delay line with a speci c arrival time (time-tag) with respect to the FPGA clock signals (186, 187). When implementing a TDL on a FPGA, a speci c CLB element known as CARRY is used as a fundamental delay block to build the tapped delay line. A TDL is indeed assembled by interconnecting multiple CARRY elements of different logic cells. In a ash TDC, the output of each delay block gets then connected to the CLB ip- op (DFF) component (Fig. 2.6 top pane). The DFF can retain either a boolean '0' or '1' depending on the digital-data input value.

Figure 2.6TDL-TDC . Schematic representation of a delay line TDC constituted by (i) a tapped-delay line, (ii) a DFFs latch barrier and (iii) thermometer-to-binary encoder (top); working principle of an TDL-TDC: the input signal propagates along the TDL until reading event occurs. By knowing the reciprocal relationship between the CARRY delay value and the distance the input signal covered before the STOP event occurred, it is possible to convert the digital measurement of the travelled distance into a time measurement (bottom).

Working principle

Assuming that the START signal holds a boolean '0' (false) at the steady state and '1' (true) after a triggering event (such as the arrival of a single-photon), then the digital-logic high value '1' will propagate through each of the tapped delay line elements. Consequently, the data input of each DFF $b(t_n)$ will also change from '0' to '1'. The START signal continues to propagate freely along the TDL until a reading event occurs. This TDL reading event, known as STOP event, freezes the values of the DFFs barrier connected to the TDL elements giving out a digital reading of how far the START signal travelled along the TDL in respect to the STOP signal (start-stop time). Knowing the reciprocal relation between the CARRY delay value and by counting the number of '1's, it is possible to convert the distance the

START signal covered before the STOP event occurred into a time measurement (Fig. 2.6 bottom pane).

Limitations

TDL-TDCs yield a higher time-resolution when compared to coarse counter and SCS TDCs, but, on the other hand, lack linearity due to the intrinsic and ineradicable irregularities in the FPGA fabric. TDL-TDC nonlinearities are mainly due to: (i) different values of the delay elements used in the TDL have values that greatly differ from each other (ii) the extension of the interconnections amongst the different delay blocks which jeopardize the consistency of the connection path delay (Fig. 2.7). The former effect profoundly interferes with delay-time conversion causing a non-linear TDL-TDC response. The irregularities can be categorised in two groups:

- Ultra-wide time bins (UWB): mainly caused by the TDL crossing adjacent clock regions of the FPGA resulting in a long interconnection path between two consecutive CARRY blocks. This type of alteration corresponds to a longer delay value in the delay line.
- Zero-length time bins (ZLB): caused by the presence of CARRY elements which delay value is negligible.

Figure 2.7 Limitations of the TDL-TDC architecture . The delay elements used in a TDL have values that greatly differs one to another, moreover the interconnections amongst the different delay blocks extend across adjacent clock regions of the FPGA jeopardizing the consistency of the connection path delay and leading to an uneven time-delay representation which is affected by UWBs and ZLBs.