MICRO PHOTON DEVICES

# SPC$^3$

# Single Photon Counting Camera

© Micro Photon Devices S.r.l.
Via Stradivari, 4
39100 Bolzano (BZ), Italy
email info@micro-photon-devices.com
Phone +39 0471 051212 • Fax +39 0471 501524

SPC$^3$ User Manual Version 1.0.1 - October 2015

# Table of Contents



# Introduction

SPC$^3$ is a single photon counting camera based on a 2-D imaging array of 64 x 32 smart pixels. Each pixel comprises a single-photon avalanche diode (SPAD) detector, an analogue front-end and a digital processing electronics. This on-chip integrated device provides single-photon sensitivity, high electronic noise immunity, and fast readout speed. The imager can be operated at full resolution with a maximum frame rate of about 100.000 frames per second with negligible blind time (dead-time). Inside each pixel three independent counters are integrated. Each one of them can be independently gated through an external signal; counter 1 can also be gated by an internally generated signal (e.g. for FLIM mode acquisition). Counters 2 and 3, instead, can also count downward, with their direction controlled through an external signal.

SPC$^3$ pixels feature high photon-detection efficiency (PDE) in the visible and near-UV spectral region, and low dark-counting rates, even at room temperature. The imager is easily integrated into common optical setups thanks to a Thorlabs® SM1 thread, a C-mount mechanical adapter and a high-speed USB 3.0 computer interface. The camera is shown in Figure 1.

The camera differs from conventional Charge-Coupled Devices (CCD) or CMOS sensors because it performs a "fully digital" acquisition of the light signal. Each pixel effectively counts the number of photons which are detected by the sensor during the acquisition time.

Figure 1. SPC$^3$: Single photon Counting Camera.

# Single Photon Avalanche Diode

The SPAD is a p–n junction which is reverse-biased well above its breakdown voltage. Under this operating condition, the absorption of a single photon generates an electron-hole pair, which is accelerated by the high electric field across the junction. The energy of the charge carriers is eventually sufficient to trigger a macroscopic avalanche current of few milliamperes through the device. The SPAD biasing electronics, namely the Active Quenching Circuit (AQC), is integrated on the same silicon chip. The AQC senses the avalanche current, quenches it and resets the diode to its initial state. The avalanche is quenched by decreasing the voltage across the SPAD junction below breakdown for an adjustable time (hold-off), that ranges from few tens to few hundreds of nanoseconds. The SPAD bias voltage is then restored to the initial state, and thus the diode is ready to detect the next photon. The total time, which is required to restore the initial state of the SPAD after the detection of a photon, is named dead-time. During the dead-time, no photons can be detected. The AQC provides, additionally, a voltage pulse to an associated counter, which is integrated in each pixel.

The measurement of light signals by a SPAD has several advantages concerning the signal to noise ratio. No analogue measurement of voltage or current is needed, since the detector acts like a digital "Geiger-like" counter. It follows that no electronic noise is added by analogue to digital converters or amplifiers while measuring the signal. Additionally, the detector is less sensitive to the electromagnetic interference or the electrical noise generated by external equipment, differently from charge coupled devices.

The primary noise sources for SPADs are:

1) *Dark counts*. This noise source comprises all processes which can start an avalanche across the SPAD junction but which are not caused by the detection of a photon. The typical source of dark counts is the thermal carrier generation process. Dark counts are the dominant noise source for the SPADs of the SPC³.

2) *Afterpulsing*. The detection of a photon can trigger, with a certain probability, an additional detection event within few microseconds. This spurious event is caused by charge carriers, that flew through the junction during the avalanche, remained trapped and are then subsequently released. These carriers are accelerated by the high electric field across the junction and might trigger another avalanche like the photo-generated electron-hole pairs, when released after the dead-time. The afterpulsing probability depends on the detector dead-time and it is usually reduced to about 1-2% at the normal operating conditions. Longer dead-times decrease the afterpulsing probability.

3) *Cross-talk*. During the avalanche process, photons are emitted by hot carriers. These can propagate through the silicon and trigger a detection event in SPADs which are placed in close proximity. Practically, the Cross-talk probability is very low among the pixels of the $SPC^3$ (about $10^{-5}$), due to the large distance between the SPADs. Its contribution to the total noise is mostly negligible.

Since the read-out noise of CCD or CMOS cameras is normally slightly higher than one electron (per integration time) and since the dark counting rates of the SPAD pixels of the SPC3 is about one hundred per second, it follows that the optimal operating condition for the camera is at fast or moderate frame-rates. Particularly, the dark-counts contribution is low with exposure times of about 0.1 s. By reducing even further the integration time, the dark count noise becomes negligible.

Due to the dead time, the SPAD has a linear working range, i.e the number of detection events within a defined time period (*T*) depends linearly on the illumination intensity, only at low or moderate photon fluxes. At large photon fluxes the number of detected photons deviates from linearity and saturates to a constant value, which is the reciprocal of the dead-time ($T_{dead}$). As a matter of fact, since the diode is off for a period equal to $T_{dead}$ every time it detects a photon, the maximum count rate happens when the detector oscillates with a period $T_{dead}$. Supposing $N_{imp}$ the number of impinging photons on a SPAD active area during an integration time T, $N_{counted}$ the photons counted by the detector, and taking into account the PDE, $N_{counted}$ is equal to $N_{imp}$ reduced by the PDE and multiplied by the ratio of the actual integration time divided by the set integration time T :

$$N_{counted} = N_{imp} * PDE * \frac{T - N_{counted} * T_{dead}}{T} = N_{imp} * PDE * (1 - N_{counted}\frac{T_{dead}}{T})$$

$$N_{counted} = \frac{PDE * N_{imp}}{1 + PDE * N_{imp} * \frac{T_{dead}}{T}} \qquad eq.1$$

By expressing $N_{imp}$ as a function of $N_{counted}$ one obtains also:

$$N_{imp} = \frac{1}{PDE} * \frac{N_{counted}}{1 - N_{counted} * \frac{T_{dead}}{T}} \qquad eq.2$$

The total number of detected events is thus smaller than the total number of photons which cross the p-n junction, because of the saturation effect and the PDE. When $N_{imp} * \mathrm{PDE} = \frac{T}{T_{dead}}$, the number of counted events is already reduced of 50%. Since a SPAD is blind for $T_{dead}$ after each triggering, it is also clear that in a time period *T*, the maximum number of photons that can be counted is :

$$N_{counted,max} = \frac{T}{T_{dead}} \qquad eq.3$$

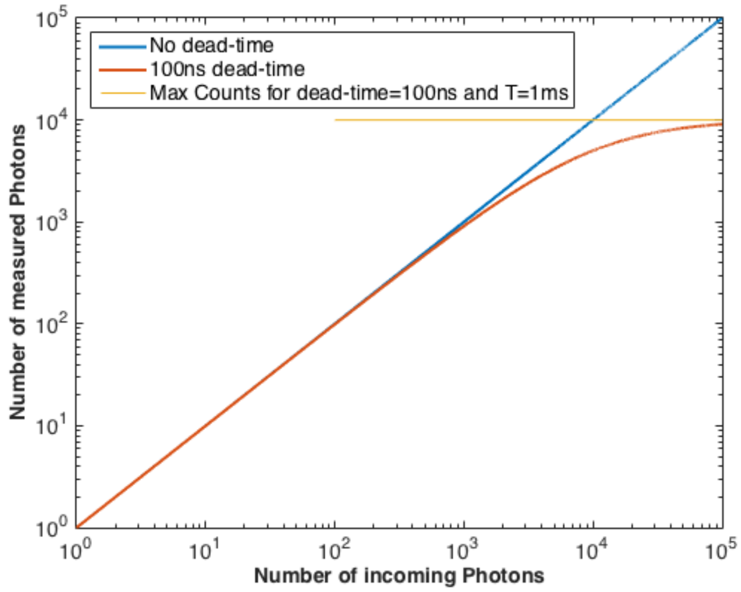



**Figure 2. Number of measured photons in an image of 1 ms exposure and dead-time of 100 ns as a function of the number of incoming photons (PDE = 100% for simplicity).**

Let's suppose for example an exposure time T = 1 ms, a PDE = 50% for simplicity and a dead-time $T_{dead}$ = 100 ns. In this case, the maximum number of photons that can be counted (or more in general the maximum number of SPAD triggerings) is 10'000. In our example also $N_{imp} * \mathrm{PDE} = \frac{T}{T_{dead}}$ corresponds to 10'000; this means that when a photon flux of 20'000'000 photons/s hit the detector, during the integration time *T*, only 20'000 ($N_{imp}$) photons cross the active area but only 10'000 can be detected due the PDE of 50%, and finally only 5'000 events are, on average, counted ($N_{counted}$) because of saturation. See, for example, Figure 2 that shows the number of counted photons as a function of the number of the impinging photons for an exposure time T = 1 ms, a dead-time of 100 ns and a PDE of 100% for simplicity.

## *Further readings*

M. Ghioni, A. Gulinatti, I. Rech, F. Zappa, S. Cova, "Progress in Silicon Single-Photon Avalanche Diodes", *IEEE Journal of Selected Topics in Quantum Electronics*, **2007**, 13, 852-862.

D. Bronzi, F. Villa, S. Tisa, A. Tosi, F. Zappa, D. Durini, S. Weyers, W. Brockherde, "100 000 Frames/s 64 × 32 Single-Photon Detector Array for 2-D Imaging and 3-D Ranging", *IEEE Journal of Selected Topics in Quantum Electronics,* **2014**, vol.20, no.6, pp.354-363.

M. Vitali, D. Bronzi, A.J. Krmpot, S.N. Nikolic, F.J. Schmitt, C. Junghans, S. Tisa, T. Friedrich, V. Vukojevic, L. Terenius, F. Zappa, R. Rigler, "A Single-Photon Avalanche Camera for Fluorescence Lifetime Imaging Microscopy and Correlation Spectroscopy", *IEEE Journal of Selected Topics in Quantum Electronics*, **2014**, vol.20, no.6, pp.344-353.

# SPC3 hardware characteristics

## *Electrical connections*

The SPC[3] has power and USB connections on one side, and a multi-coaxial connector on the other side for various control signals, as shown in Figure 1 and Figure 3. SPC[3] is provided with a cable adapter from the multi-coaxial connector to standard SMA connectors as shown in Figure 4. A description of the input and the outputs can be found in Table 1. The Electrical specifications of the input and outputs are reported in Table 2.

**Table 1. SPC3 Inputs and Outputs description.**

| | |
|---|---|
| USB | High-speed USB 3.0 connector. It is recommended to use short USB cables (< 2 m). |
| $+12V_{DC}$ | Jack for connecting the provided +12V power supply. |
| SYNC OUT | Coaxial output which can drive 50 Ohm terminated transmission lines. A 50 Ohm DC load termination is required. Electric pulses of 3.3V LVCMOS are generated at this output for synchronizing the camera with any external device. The SPC[3] can be configured by the user to output the internal 50 MHz *reference* clock, synchronised with the internal 100MHz *master* clock, for synchronizing any external instrument (such a laser) with the internal generated gate. The same output can be configured also to generate a signal (pulse) synchronous with the start of each hardware integration frame. The delay between the actual start of the integration frame and the rising edge of this pulse is shown in Figure 5. For more details on gated operation, see the corresponding paragraph in the *SPC[3] Operation* section. |
| TRIG IN | This coaxial input requires a 3.3V pulse (5V tolerated) of minimum duration of 40 ns to start the camera acquisition using a signal from an external device (e.g. a shutter, a laser, etc.). The first frame starts between 20ns and 40ns after the TRIG IN rising edge. Since the TRIG IN signal is asynchronous respect to the internal clock, such delay is of course not-deterministic (unless it is synched with the SYNC OUT signal). The input impedance is 50 Ohm. See Figure 5 for reference. |
| GATE IN 1<br>GATE IN 2<br>GATE IN 3 | Active-low inputs with 50 Ohm DC input impedance. Each input controls one of the three in-pixel counters. When GATE IN is '1', the incoming photons are not counted by the measuring electronics. 3.3V LVCMOS signals are accepted but the inputs are 5V tolerant. The minimum duration of a GATE IN pulse is 5 ns. The actual internal gate is delayed by about 15ns. |
| DIRECTION | Input to select the counting direction of counters 2 and 3. The input has a 50 Ohm DC input impedance; 3.3V LVCMOS signals are accepted but the input is 5V tolerant. A low value means UP counting. DIRECTION timings TBD. |

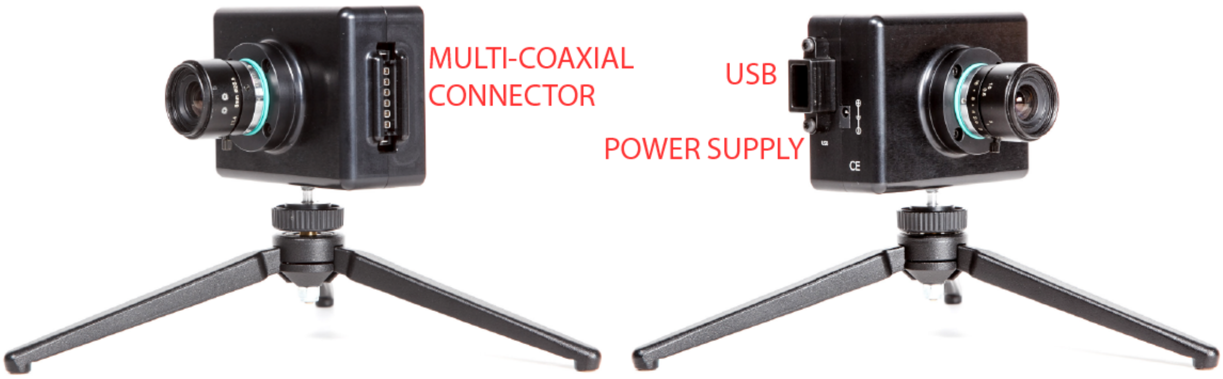



Figure 3. Camera side-views: hardware connections.

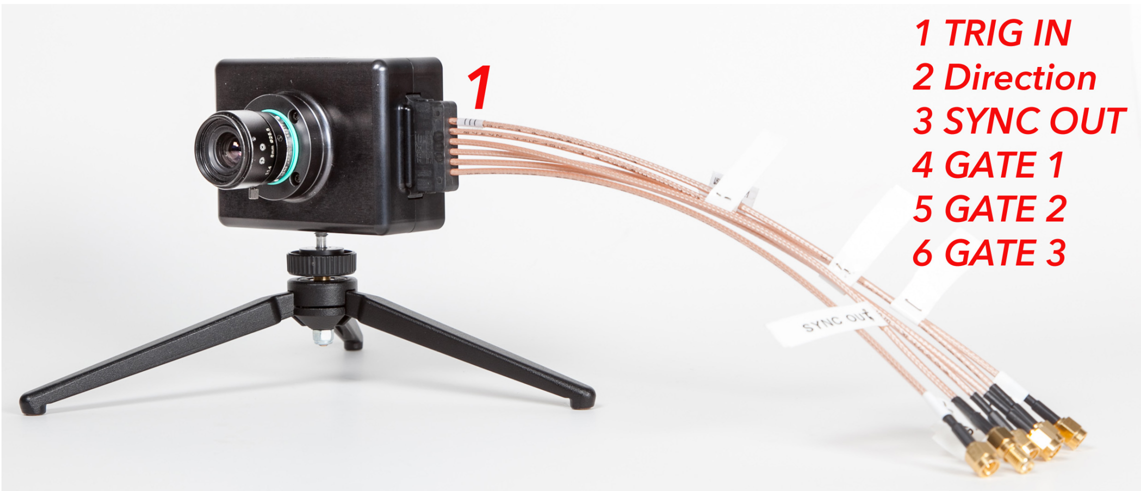



Figure 4. Camera with the cable adapter and the SMA output description.

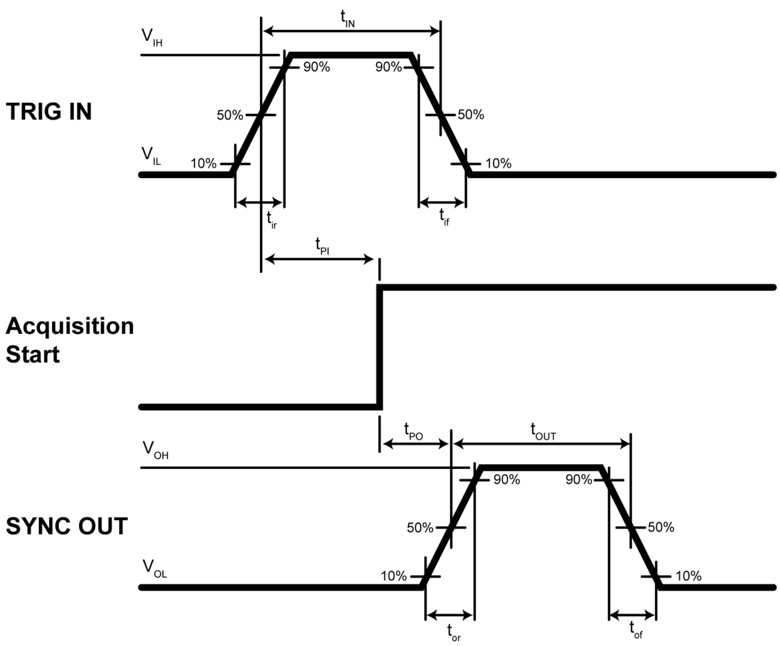



Figure 5. Timing of TRIG IN and SYNC OUT signals with respect to the actual start of the acquisition.

**Table 2. Electrical Characteristics of the SPC3 measured at room temperature (25°C)**

| Symbol | Parameter | Signal | Min | Typ | Max | Unit |
|---|---|---|---|---|---|---|
| $V_{IH}$ | Input logic-high voltage (5V tolerant) | TRIG IN, GATE, DIRECTION | 2.4 | | 3.3 | V |
| $V_{IL}$ | Input logic-low voltage | TRIG IN, GATE, DIRECTION | 0 | | 0.8 | V |
| $V_{OH}$ | Output logic-high voltage (50Ω load) | SYNC OUT | | 2.7 | | V |
| $V_{OL}$ | Output logic-low voltage (50Ω load) | SYNC OUT | | 0 | | V |
| $t_{PI}$ | Propagation delay from TRIG IN to acquisition start | TRIG IN | 20 | | 40 | ns |
| $t_{IN}$ | TRIG IN pulse duration | TRIG IN | 40 | | | ns |
| $t_{PO}$ | Propagation delay from acquisition start to SYNC OUT | SYNC OUT | 5 | | 10 | ns |
| $t_{OUT}$ | SYNC OUT pulse duration (frame sync mode) | SYNC OUT | | 40 | | ns |
| $t_{ir}$ | Input rise time (10%-90%) | TRIG IN, GATE | | | 5 | ns |
| $t_{if}$ | Input fall time (10%-90%) | TRIG IN, GATE | | | 5 | ns |
| $t_{or}$ | Output rise time | SYNC OUT | | 1 | | ns |
| $t_{of}$ | Output fall time | SYNC OUT | | 1 | | ns |
| $t_{gate}$ | GATE pulse duration | GATE | 5 | | | ns |
| $t_{gate\_delay}$ | GATE delay | GATE | 10 | 15 | 20 | ns |
| $t_{dir\_delay}$ | DIRECTION delay XXX | DIRECTION | XX | | XX | ns |
| - | Internal reference clock - frequency | SYNC OUT, internal | | 50 | | MHz |
| - | Internal reference clock - duty | SYNC OUT, internal | | 50 | | % |

## *Camera mechanical dimensions*

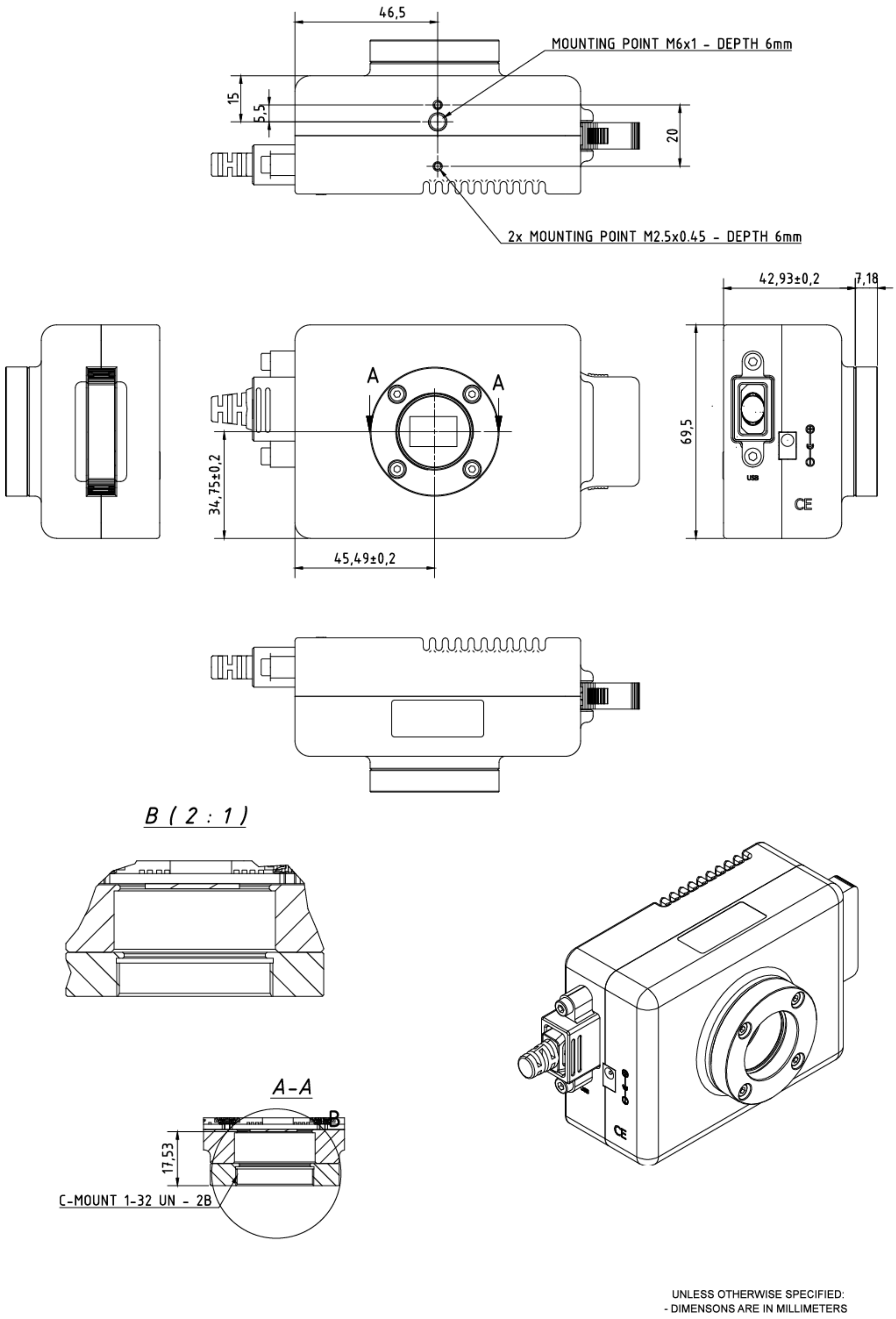



**Figure 6. SPC3 mechanical dimensions. All dimensions in mm.**

# SPC$^3$ Operation

The SPC$^3$ camera features three different modes of operation that can be optimised through the use of several working parameters, all controllable from a computer using either the delivered SDK library or the provided Windows® PC software. The following paragraphs describe in detail all these features.

## *Standard operation*

The camera is operated in its *standard* mode when each pixel is simply used to count photons in a user defined integration time and the counters are not gated (see the next paragraph for details). When the SPC$^3$ is operated in this mode, three different acquisition modes are possible:

- *Live acquisition*: single images from the camera are acquired and downloaded to the computer. Since in this mode it is the PC that continuously asks for new images, the time between two images can not be precise and strongly depends on how the PC software behaves and on the PC's current work load. This mode should not be used for actual scientific acquisition, but it is instead useful for acquiring a low frame rate "live" movie (i.e. real-time), for instance when aligning the camera in the optical setup or adjusting the acquisition parameters with the current illumination conditions. Considering the practical limitations of the USB connection when transferring small data blocks, typical achievable frame-rates are in the range of 50-100 fps (actual values depend on the performance of both the PC hardware and the software employed). As a consequence, the PC requests of live images should be limited to a maximum of 1 every 10 ms or longer time.
- *Snap*: a sequence of images is acquired and stored into the internal memory of the camera. The dead period between subsequent frames (inter-frame dead-time) is less than 10 ns, and the frames' timing is accurately determined by the internal clock. Once the required number of frames is measured, the electronic interface transfers the data to the computer. The images can then be displayed and saved on the hard-drive. This mode can not be used for live acquisition, since all data will be downloaded to the PC only at the end of the entire snap, which can last few seconds. Instead, this approach allows data acquisitions at the maximum frame-rate possible, since it does not depend on the actual throughput of the USB link or PC save speed. Snap size is limited by the capacity of the internal memory (128 MiB). For more details on how to calculate snap size, see the paragraph on *Camera Configuration*. If you need to acquire more data you should use *Continuous Acquisition* mode, as described below.
- *Continuous Acquisition:* a continuous acquisition of data between a START command and a STOP command from the PC is performed. Once the acquisition is started, the internal memory is used as

a buffer, and data should be continuously read by a PC. In case the internal memory gets full because the PC fails to download the data swiftly enough, data loss occurs and an error is issued. The maximum achievable frame rate strongly depends on PC performance (specially on the HDD write speed). Thanks to USB 3.0 it is possible to acquire the full array at the maximum speed (about 100kfps). However, in order to save all generated data at this high throughput it is also crucial that the employed mass-storage unit is very fast (see the paragraph on Camera Configuration for more details on how to calculate actual data rates). SSD storage units are thus recommended. If your PC can not sustain such a high throughput you will have to reduce the frame rate or to save only half the array. If this is not possible you must resort to the *Snap* mode, at the expense of the experiment duration.

In both *Snap* mode and *Continuous Acquisition* mode it is possible to trigger the actual start of the acquisition with an external signal.

In all three modes it is possible to output a pulse synchronous with the start of each integration time. It is also possible to internally subtract a background image previously stored into the camera.

## *Gated operation*

Normally, the counters of each pixel of the SPC³ will just continuously count all the pulses generated by the SPADs. This operating mode is called *free-running* and it is the default operation mode. Anyway, each of the three integrated binary counters which register the detected number of photons in each pixel can be *enabled* or *disabled* by a *gate* signal. This operating mode is called *time-gating*.

The *gate* signal for counters 2 and 3 is provided only by the two coaxial inputs GATE IN 2 and GATE IN 3. The *gate* signal for counter 1 instead is the logical OR between two independent digital signals: GATE IN 1, i.e. one of the camera coaxial inputs, and *Software gate,* generated internally by the SPC³ electronics, which can be set by the user through the provided software. Each of the three external GATE IN signals can be periodic or aperiodic and it will be applied asynchronously and directly (through the OR gate) to the SPADs. The *Software Gate* is a periodic digital signal, which is synchronized with the internal *Reference clock* (20 ns period). The user can set the *Delay*, i.e. the time shift between the rising edge of the *Reference clock* and the high-to-low transition of *Software gate*, and the parameter *Width*, which defines the duration of the "*counter-enabled*" *Software gate* pulse. Essentially, the *Software gate* generates a periodic gate signal of a given *Width* and *Delay* from the reference clock. Note that the *Width* is the duration of the *Software Gate's* low state as shown in Figure 7.

The *Width* of the *Software Gate*, which is generated by the control electronics, can be varied in the range 0 ÷ 100% of the reference clock, in steps of 1%. Actually, during a factory calibration, the Gate width has been internally discretized with 1000 points over the full range. In this way, whenever one of the 100 nominal selectable values is requested by the user, the SPC$^3$ camera is set to the closed possible one, i.e. the one with the smallest possible error. *Note that, however, values below 2 ns could be unreliable*. Particularly the minimum achievable Gate signal is about 1.5ns. The actual set value can be reported to the user by the software. The Delay can be varied in the range -40% ÷ +40% of the Software Gate period, in nominal steps of 0.1%, corresponding to 20 ps. The actual gate-delay step can actually vary depending on the used camera, however an internal calibration ensures that the integral non linearity in delay setting is always less than +/- 20ps. An initial offset may also be present. This offset is on purpose not calibrated, since it will sum up with other setup-depended offsets (e.g. from different cable lengths). The overall system offset can be evaluated through optical measurements employing a short-pulse laser (i.e. less than 100ps pulse width).

When the SPC$^3$ is operated in gated mode, the same three acquisition modes of the *standard operation* are possible, this time in conjunction with the use of the three gates.

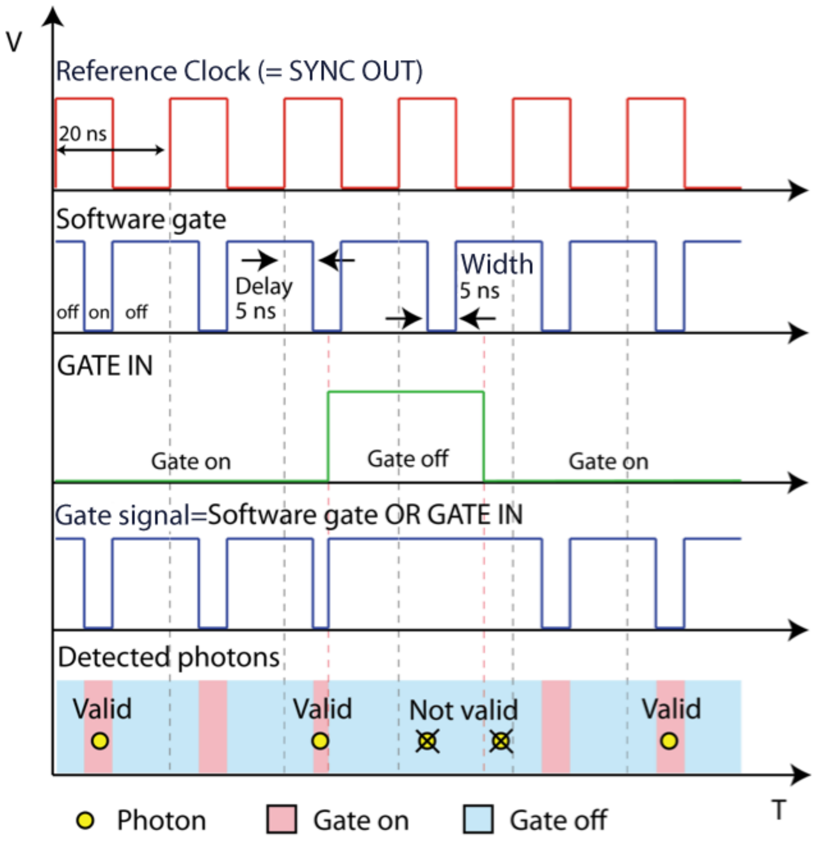



**Figure 7. Hardware gating example: Delay = 5 ns and Width = 5ns (both of 25% of the Reference Clock period). The reference clock has been sent to the SYNC OUT output of the camera. Additionally, a GATE IN signal was provided by the user. Only the photons, which are detected when the "Gate signal" is on, are counted. Example valid for counter 1; for counter 2 and 3 the example remains the same without the software gate. In such case only the 4th photon would not bet detected.**

## *FLIM Operation*

Fluorescence Lifetime IMaging (FLIM) is an imaging technique based on the measurement of the exponential decay rate of the fluorescence from a fluorescent sample, rather than of its emission intensity. The fluorescence lifetime of a molecule is a measurement of the rate of decay of the emission, which is a property of the individual single molecule, and thus it is unaffected by changes in probe concentration or excitation intensity. FLIM can be used for instance as an imaging technique in confocal microscopy and two-photon excitation microscopy.

A typical application of FLIM is the study of Förster Resonance Energy Transfer (FRET), the mechanism of energy transfer between two chromophore molecules with the emission band of one overlapping the absorption band of the other. When this two components are in close proximity, the first one, called donor, initially in its electronic excited state may non-radiatively (without emission of the light) transfer the energy to the second one, called the acceptor. The result is the quenching of the donor fluorescence, thus the decrease of the donor emission lifetime. The efficiency of the energy transfer is inversely proportional to the sixth power of distance between donor and acceptor, making the effect noticeable only at distances shorter than 10 nm. FRET is used to determine the proximity in the nano-meter range between proteins or other elements of interest associated with suitable fluorophores labelled as donors and acceptors. Such measurements are used as a research tool in fields such as biology and chemistry. Different ways of performing FRET measurement exist. The obvious problem with the intensity-based steady-state FRET measurements is that the emission band of the donor extends into the emission band of the acceptor and the absorption band of the acceptor extends into the absorption band of the donor. Furthermore, the concentrations of the donor and acceptor and the fraction of donor molecules linked to an acceptor molecule are variable and unknown. All this makes the intensity-based FRET measurements requiring calibration with the samples containing only donors and acceptors, or measurements in which the second step is the destruction of acceptor by photo-bleaching, obtaining the FRET measurement as the relative increase of donor fluorescence intensity. The FLIM-based FRET measurement has the advantage that the results are obtained from a single lifetime measurement of the donor and are not affected by the changes of the sample concentration, excitation intensity and other factors that limit the intensity-based FRET measurements.

FLIM images are most often obtained by employing the Time Correlated Single Photon Counting (TCSPC) technique and a point detector (such as a PMT), which is then scanned in order to reconstruct the decay histogram for each image pixel. The exponential decay model is fitted to each pixel histogram in order to determine the lifetime. The colour of each image pixel represents the determined lifetime, giving the possibility to obtain images with contrast between materials with different decay rates even if they fluoresce at the same wavelength, or, on the other hand, identify two region of the same material even if they have different intensity. The use of an array of SPADs has major advantages for imaging applications since, as opposed to single pixel systems, it does not require any scan of the sample. A second major advantage of the presented pixel structure concerns the short dead-time, which has a lower limit of about 50 ns, thus allowing the counting of very high photon fluxes.

The embedded short gate capability enables the use of $SPC^3$ as a FLIM camera even though the employed SPAD imager chip is only counting the detected photons and it is not time-tagging them. In fact, it is possible to employ the gate in order to implement a time-gated FLIM detection system. In time-gated FLIM the decay of the fluorescence is reconstructed by repetitively counting the number of detected photons in short time windows that are progressively shifted with respect to the excitation laser pulse, as shown in Figure 8. In order to perform this type of measurement, the SYNC_OUT output form the $SPC^3$ camera has to be connected to the trigger input of a pulsed laser source able to generate optical pulses with width of at most few hundreds of picoseconds. Such laser is then used to excite the fluorescence in the sample under test. The internally generated *Gate* signal activates the counters after the generation of the laser pulse for the time defined by *gate width.* Accordingly, the fluorescence decay kinetics is measured by changing *gate shift* (position) over time. Both *gate shift* and *gate width* have optimal values depending on the lifetime of the excited state of the fluorescent molecules and on the imaging frame-rate.

In order to speed-up FLIM acquisitions and increase the frame rate, the $SPC^3$ includes an automatic FLIM mode in which the internal gate signal is automatically generated with user-defined step-width and shifted of the user-desired number of steps.

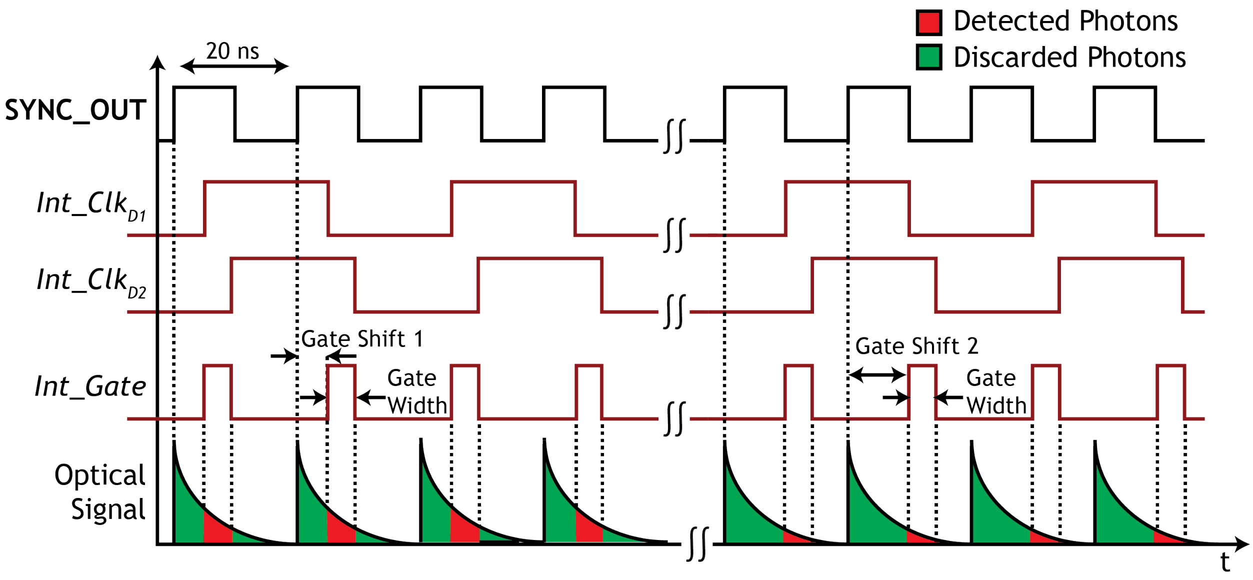



**Figure 8. Optical waveform reconstruction using the Gated-FLIM approach with the SPC³ camera.**

Each "FLIM acquisition" is thus composed by a sequence of FLIM elementary (step) frames, one for each desired gate position (step), each one consisting of an acquisition with the same exposure parameters of a standard acquisition. FLIM mode acquisitions are possible in Snap mode and Continuous Acquisition mode whereas a real Live mode in not available, since each FLIM acquisition is composed by several elementary frames. However, the VisualSPC$^3$ software (see below) offers a seamlessly equivalent Live mode which is internally implemented performing repetitive Snap acquisitions.

Due to the time required for internal gate shifting (which is also dependent on the shift parameters), the time required for a full FLIM acquisition is not simply the sum of the integration time of each step and it is, instead, conveniently calculated by the camera software and reported to the user. A reference clock inside the camera guarantees that every FLIM acquisition starts only after this time is elapsed, thus ensuring the isochronicity of FLIM acquisitions, and allowing the recording of "FLIM movies".

Exactly as for the *gated operation,* the gate signal is generated synchronized with the internal 50MHz *reference clock*. Its *width* can be varied in the range 0 ÷ 100% of the reference clock period (20 ns), in steps of 1%, while the *gate shift* can be varied in the range -40% ÷ +40% of the same period, in nominal steps of 0.1%, corresponding to 20 ps. Of course, in order to make the Gated-FLIM work, the external laser has to be synchronised with the gate and thus, in case of FLIM, the SYNC OUT outputs the internal reference clock. Finally, as shown in Figure 8, since the reference clock period is 20ns, the nominal maximum "observation" time window is also 20ns. Actually, since the gate shift range is -40% ÷ +40%, the gate can be moved from 2 ns to 18 ns and thus the actual maximum observation window is 16 ns. The reason for the reduced range is due to the fact that for shifts longer than +/- 40% of period, the shift accuracy is not satisfactory and thus the SPC$^3$ FPGA does not allow their selection.

The actual width and phase of the gate signal obtained for a given set of parameters may change among different SPC3 units, due to fabrication tolerances of the FPGA. That's why the gate width is calibrated by MPD before delivering the camera.

The initial position of the gate in a FLIM sequence is also internally calibrated and has an accuracy better than +/- 20ps plus a constant offset (see also paragraph on *Gated Operation*). This *shift-offset* is not calibrated since in case of a FLIM experimental set-up what is really important to measure is the full offset of the gate signal with respect to the laser pulse. This offset is not only determined by the internal *shift-offset* due to the camera, but also (and probably largely) by the delays introduced by the used external interconnection cables and the laser itself. A calibration of the actual gate shift with respect to the laser excitation is therefore necessary *after* the camera is mounted into the measurement setup. This can be easily done by directly shining the laser onto the array, without interposing any fluorescent sample or filter, and performing a full measurement over the entire 20 ns shift range. By inspecting the measurement and finding the laser peak, it is possible to evaluate the shift between the actual laser pulse and the internal reference clock. Actually it is not even important to measure it. What needs to be done is simply the adjustment of the cables' length from the SYNC_OUT of the camera to the TRIG_IN of the laser in order to place the laser pulse at the beginning of the FLIM observation time window. In this way the fluorescence decay will be centred inside the 16 ns FLIM window. This would also prevent any folding of the fluorescence signal and the associated measurement distortion. Of course since the first and the last 2 ns of the FLIM window are ignored, the laser pulse is not seen when it falls there. If this happens, making 1 m longer of shorter the cable that connects the camera to the laser will make the laser pulse reappear in the FLIM observation window. Such calibration could be useful also to zero any skew among pixels but, in many applications, like the FLIM, it is usually not needed; should it be, the SPC3 user can contact MPD at the following email address for more details: imaging@micro-photon-devices.com

Finally, a factory calibration is performed also on the actual average gate-shift bin (i.e. minimum gate shift): this value is reported to the user through the provided software, and it should be used as a scale factor on the time axis of the reconstructed FLIM waveform.

FLIM data acquired can be either saved on a multipage TIFF with embedded acquisition metadata according to the OME-TIFF format or in the proprietary SPCF format. For both formats, image data is composed by a set of images following a "FLIM first, time second scheme", i.e. with the following frame sequence: 1$^{st}$ gate shift of 1$^{st}$ FLIM measurement, 2$^{nd}$ gate shift of 1$^{st}$ FLIM measurement, ..., n$^{th}$ gate shift of 1$^{st}$ FLIM measurement, 1$^{st}$ gate shift of 2$^{nd}$ FLIM measurement, 2$^{nd}$ gate shift of 2$^{nd}$ FLIM measurement, ..., n$^{th}$ gate shift of 2$^{nd}$ FLIM measurement, etc.

OME-TIFF file could be opened with any image reader compatible with TIFF file, since metadata are saved into the Image Description tag in XML format. In order to decode OME-TIFF metadata, it is possible to use free OME-TIFF readers, such as OMERO or the Bio-Formats plugin for ImageJ. For more details see the OME-TIFF web site: http://www.openmicroscopy.org/site/support/ome-model/ome-tiff/. OME-TIFF metadata include the ModuloAlongT tag, which allows the processing of FLIM data with dedicated FLIM softwares such as FLIMfit (see http://www.openmicroscopy.org/site/products/partner/flimfit). A tutorial on basic usage of FLIMfit is available at http://help.openmicroscopy.org/flimfit.html. In order to import OME-TIFF file generated by SPC3 camera, simply select "Load FLIM data…" from File menu. For further support on FLIMfit please contact directly its developer.

SPCF file are binary files composed by a header with acquisition metadata followed by raw image data, containing the 8/16 bit pixel values in row-major order. SPCF file can be read using the provided ImageJ/Fiji plugin. In addition, since the format is fully documented in SPC3-SDK documentation, users can directly read data from their own code.

## Further Readings


1. Joseph R. Lakowicz. Principles of Fluorescence Spectroscopy 3rd edition. Springer (2006).
2. J. Pawley, *Handbook of Biological Confocal Microscopy, 3rd Edition.* New York: Plenum Press, 2006.
3. T. W. J. Gadella, Ed., *FRET and FLIM Techniques, Volume 33*. Amsterdam: Elsevier Science, 2008.
4. S. P. Chan, Z. J. Fuller, J. N. Demas, and B. A. DeGraff, "Optimized gating scheme for rapid lifetime determinations of single-exponential luminescence lifetimes." *Anal Chem*, vol. 73, no. 18, pp. 4486–4490, 2001.
5. D. D.-U. Li, S. Ameer-Beg, J. Arlt, D. Tyndall, R. Walker, D. R. Matthews, V. Visitkul, J. Richardson, and R. K. Henderson, "Time- domain fluorescence lifetime imaging techniques suitable for solid-state imaging sensor arrays." *Sensors*, vol. 12, no. 5, pp. 5650–5669, 2012.


# Acquisition parameters

## Integration time

Each pixel of the SPC3 camera integrates three 9-bit binary counters (two of them with up-down capability), which measure the number of photons detected during a specified *Hardware Integration Time (HIT).* The *HIT* is thus the time during which the impinging photons are counted without resetting the integrated counters, i.e. the time from when these counters are cleared and started to the time when they are latched and read. It can range from the *Hardware Readout Time (HRT)* of the array to about 0.65 ms. The *Hardware Readout Time* of the array is proportional to the number of counters ($N_{counters}$) in use and it always corresponds to the inverse of the frame-rate. In other words the HRT is the minimum time needed to read the counters in use for all the pixels. HRT value is given by:

$$HardwareReadoutTime = N_{counters} * (5\ ns * N_{pixel} + 160\ ns)$$

In case of use of a single counter per pixel, this time is equal to the time needed to read the 2048 pixels and is 10.40 µs; for 2 counters it is equal to 20.80 µs and for 3 counters it is equal to 31.20 µs. It must be also noted that even when saving only half the array, in order to reduce the mass storage bandwidth requirement, the readout will be carried out for the entire imager and so the *Hardware Readout Time* will not be reduced.

It is also possible to increase the total integration time by internally (inside the camera's internal FPGA) accumulating several *Hardware Integration Time*, in order to obtain the so called *Frame Exposure Time.* This is a viable solution since the SPC3 has no readout noise, thus there is no penalty in repeating the readout, apart for a negligible dead-time (or inter-frame dead-time) of less than 10 ns between adjacent *Hardware Integration Time.* The number of *Hardware Integration Times* that are accumulated to obtain a *Frame Exposure Time* can range from 1 to 65534. A beneficial side effect of accumulating several *Hardware Integration Times* instead of using a longer *Hardware Integration Time* is that the equivalent dynamics of the internal counter is increased. In fact, since each *Hardware Integration Time* can accommodate up to 511 photon detections*,* 2 *Hardware Integration Time* can hold 1022 detections, 10 *Hardware Integration Time* can hold 5110 and so on.

Finally, it is possible to employ the internal detector gating capability of Counter 1 in order to achieve an *Equivalent Hardware Integration Time* smaller than the *Hardware Readout Time*. This is possible by gating-off the detector during a portion of *Hardware Integration Time. Equivalent Hardware Integration Time* can be as short as 10 ns. Please note however, that using an *Equivalent Hardware Integration Time* shorter than the *Hardware Readout Time* results in a corresponding increase in the inter-frame dead-time, by an amount equal to the difference between the *Hardware Readout Time* and the chosen *Equivalent Hardware Integration Time*. Since in this particular working mode the gate is controlled directly by the camera, it is not recommended to apply any external gate signal or to change the internal gate settings, either with the PC software or with the standard gate-control procedures provided in the SDK. Particularly the new gate settings will overwrite the already applied ones thus interfering with the *Equivalent Hardware Integration Time* generation process.

In order to configure these three parameters, the $SPC^3$ can be set into two different modes. In *normal mode* the camera settings are constrained so that the *Hardware Integration Time* is equal to the *Hardware Readout Time,* in order to avoid or at least reduce to a minimum the probability of the occurrence of an overflow of the integrated counters. In fact, the internal 9-bit binary counters can overflow at long *Hardware Integration Times* and thus induce artefacts in the detected image.

As already shown, the maximum number of photons $N_{max}$ that can be counted per second, given a dead time of $T_{dead}$, is:

$$N_{max} = \frac{1}{T_{dead}} (counts/second)$$

thus the maximum number of photons that can be counted during a *Hardware Integration Time* equal to T is:

$$N_{max-HIT} = \frac{1}{T_{dead}} T$$

For instance, when using a single counter, T is equal to 10.40 µs and with the minimum $T_{dead}$ of 50 ns, the maximum counted photons is:

$$N_{max-HIT} = \frac{T}{T_{dead}} = \frac{10.40\ \mu s}{50\ ns} \cong 208 < 511$$

thus, any overflow is avoided since 511 is the maximum number that can be counted by a 9-bit counter before overflowing. Instead, when using all the three counters, T is equal to 31.20 µs and with the minimum $T_{dead}$ of 50 ns, the maximum number of photons that can be counted is:

$$N_{max-HIT} = \frac{T}{T_{dead}} = \frac{31.20\ \mu s}{50\ ns} \cong 624 > 511$$

and the overflow may occur with very strong light.

As a consequence, in order to minimise the probability of incurring in overflows, or even to completely avoid them in some cases, a clever strategy is to obtain long *Frame Exposure Times* by accumulating many *Hardware Integration Times*, each equal to the *Hardware Readout Time*, directly in the SPC3. Due to the short inter-frame dead-time smaller than 10 ns, this operating mode induces only negligible signal losses and does not increase the noise (since read-out noise is not present in the SPC³ Camera).

In *advanced mode*, both *Hardware Integration Time* and *Frame Exposure Time* are user adjustable, thus particular care must be taken by the user in order to avoid artefacts due to the overflow of the integrated 9-bit counters, which is no more avoided or minimized. In addition, also the ability to achieve shorter *Equivalent Hardware Integration Time* on Counter 1, by using the internal gate generation, is enabled. Internal gate is automatically enabled when the user selects a *Hardware Integration Time* shorter than the *Hardware Readout Time*. It is then up to the user the calculus of the actual inter-frame dead-time; for this purpose, since the *Hardware Readout Time* is equal to the inverse of the frame-rate in *Snap* or *Continuous Acquisition* modes, it could be useful to have a look at the actual frame-rate by enabling and checking the Sync Out output. Please also note that when this feature is enabled, only Counter 1 will be automatically gated, whereas Counters 2 and 3 will have an actual *Hardware Integration Time* equal to the *Hardware Readout Time.* Figure 9 summarizes the relationship between Hardware Integration Time, Readout Time, Frame Exposure Time in the two working modes.

## *Dead-time time and dead-time correction*

SPAD dead-time can be selected between 50 ns and 150 ns both in normal and in advanced mode. There are about 60 discrete values that can be assumed over the full range, thus whenever a value is input by the user, the SPC3 camera is set to the closest possible value, according to factory calibration. The actual value can be reported to the user by the software.

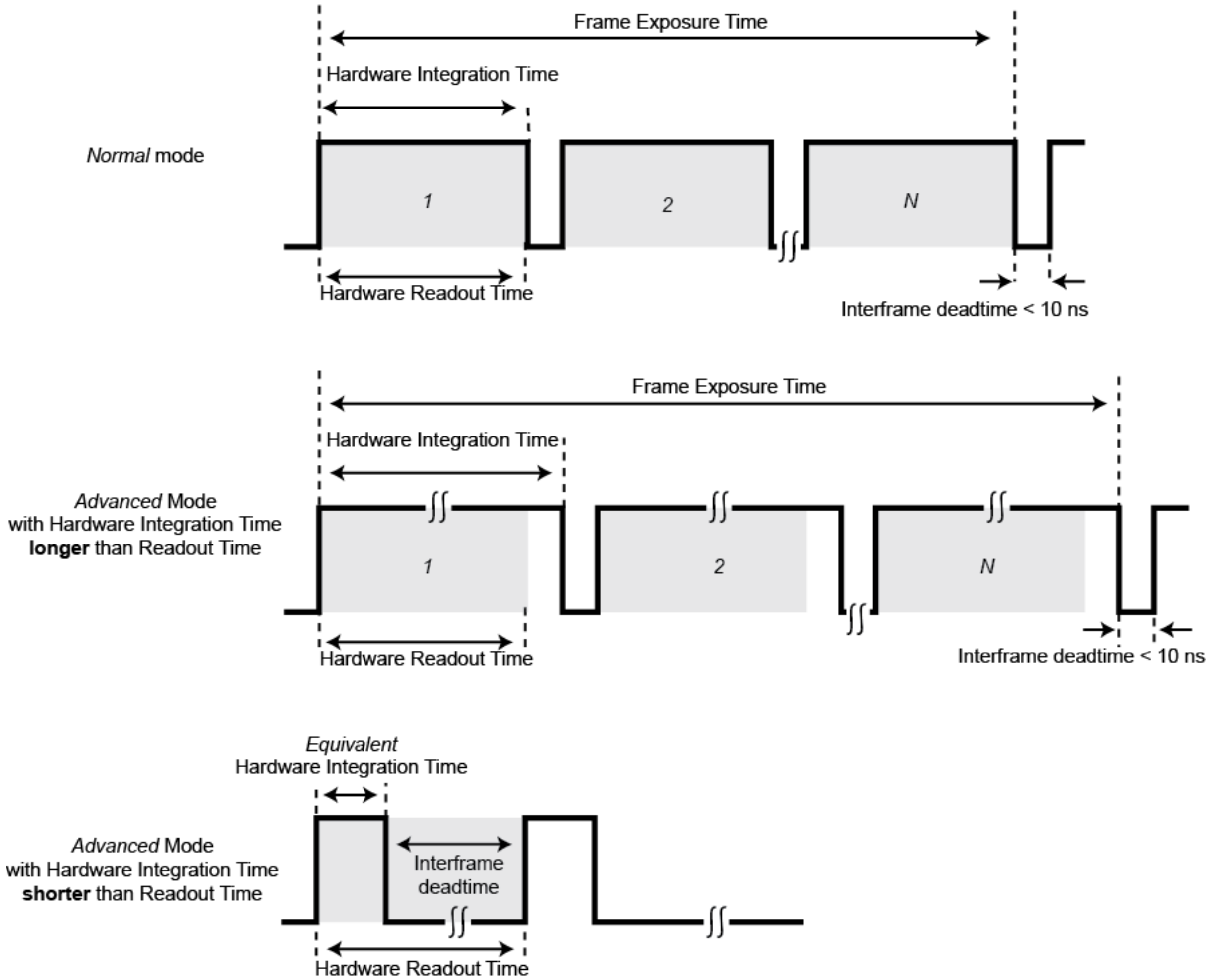



**Figure 9. Integration times and working modes.**

The dead-time strongly affects the image quality. It limits the after-pulses generated in each single pixel when set at high values, e.g. 150 ns, but it introduces a limitation on the total number of photons per second that each pixel can detect. This induces a non-linearity, when bright spots are observed (see the introductory section for further details).

It is possible to improve the quality of the image by correcting for the non-linearity introduced by the dead-time (see section "Single-Photon Avalanche Diode"). In fact, a simple operation as background subtraction might perform incorrectly when large numbers of photons per second are detected by the pixels. This occurs because, for example, a cumulated flux of 10'000 photons (5'000 signal photons + 5'000 optical background photons) might suffer a larger non-linearity in the detection than the acquisition of only 5'000 optical background photons, as predicted by Eq. 1 and shown in Figure 2. For sake of clarity let's consider, as before, 5'000 photons per integration window T both for the background and for the signal. Let's also assume T = 1 ms, a dead-time of 100 ns and a PDE = 100% for simplicity. In this situation if follows that only 3'333 photons are counted when acquiring the 5'000 background (BG) photons and only 5'000 are counted when acquiring the cumulated 10’000 photons of signal and background (SG+BG), see Eq.1 for reference. It is thus clear that subtracting the measured 3’333 BG photons from the 5’000 SG+BG photons is totally incorrect and results in the over-subtraction of the background which should have been 2’500 and not 3’333. In any case, the correct way of proceeding is to correct, by means of Eq. 2, for the dead-time effect the actual measured number of photons and obtain the real number of impinging photons (10’000 and 5’000) for both images and only AFTER perform the subtraction.

By enabling the embedded dead-time correction, the effect of the non-linearity is mostly removed and a better and faster processing of the data becomes possible. Figure 10 shows the improvement of the image quality of two “white” frames with automatic background subtraction enabled. The reference image shows a noisier pattern compared to the dead-time corrected one. This is due to an “over-subtraction” of the background, caused by dead-time induced non-linearity.

The effect is even more visible in the intensity histogram on the right of Figure 10. The histogram of the reference image deviates from an expected Gauss-like profile and shows a remarked shoulder for lower number of photons (green curve). Conversely, the corrected image is closer to the ideal Gauss distribution (blue curve).

It must be observed that the dead-time correction is only effective under a moderate illumination of the sensor. For this reason we have decided that the SW implemented dead-time correction, does compensate the non-linearity shown in Figure 2 but it does not in any case expand the counting range beyond the natural limit imposed by the hold-off time, as shown in Figure 11. As a consequence, when a strong illumination is applied, the corrected values will in any case saturate to the expected saturation level given by the current hold-off time.

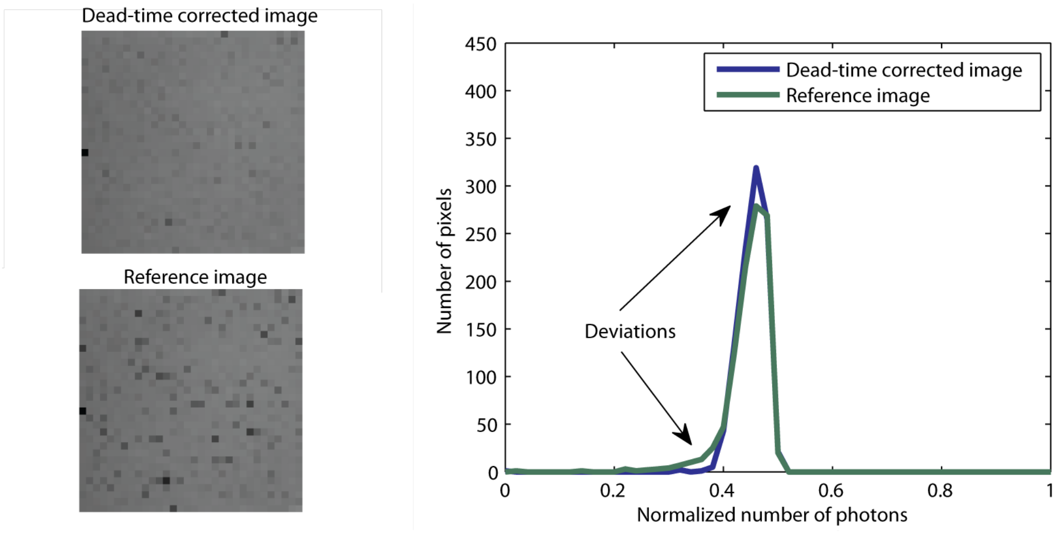



**Figure 10. Improvement of the image quality by using the dead-time correction algorithm. The intensity histograms of the two images are plot on the right.**

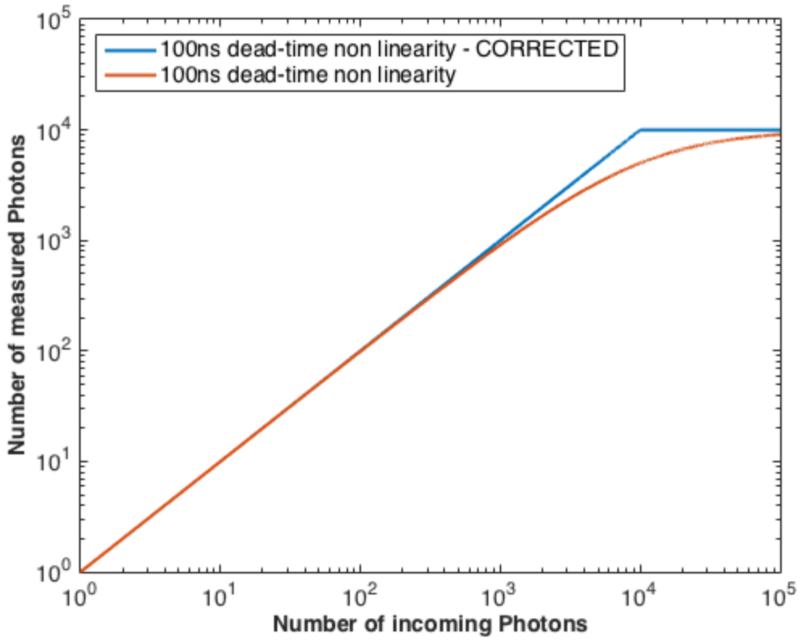



**Figure 11. Effect of the dead-time corrector in an image of 1 ms exposure and dead-time of 500 ns as a function of the number of incoming photons.**

## Background subtraction

SPC³ can perform **in-camera background subtraction**. For this purpose the user must have previously acquired a dark image (i.e. with the shutter close) *with the same parameters of the actual acquisition* (i.e. the same integration time, hold-off and gate), and then has to enable the background subtraction.

# Additional information

## Calculation of the Snap size and the Continuous Acquisition Data rate

*Integration Time* and *Half-Array Saving Mode*, directly influences the data size of a single *Snap* mode acquisition and the data rate in *Continuous Acquisition* mode.

*Snap* mode data size, in bytes, is given by:

$$Snap\_Size = N_{pixel\_Saved} * N_{frames} * \frac{BPP}{8}$$

where $N_{frames}$ is the number of frames in the Snap and bit-per-pixel (*BPP*) is 8 if there is no internal accumulation of several Hardware Integration Times and both dead-time correction and background subtraction are off, 16 otherwise. $N_{pixel\_Saved}$ is the number of saved pixel and currently can be 2048 (full array) or 1024 (half array).

*Continuous Acquisition* mode data rate, in bytes-per-second, is given by:

$$CA\_DataRate = N_{pixel\_Saved} * \frac{BPP}{8} * FrameRate$$

where

$$FrameRate = \frac{1}{Hardware\_Readout\_Time * N_{sums}}$$

being $N_{sums}$ the number of internal accumulations.

## *Camera auto-protection*

The SPC$^3$ has a protection mechanism that shuts down the SPAD high voltage when too much current is flowing through the array, for example when really high photon fluxes are impinging on the camera. Solution: Reduce the amount of light that falls on the sensor, disconnect and reconnect the camera to the host PC and restart the software.

# SPC3 Software interface – VisualSPC³ (Windows only)

The camera is provided with an acquisition and control software, which is running on Microsoft Windows operating systems: *VisualSPC³*. This software provides a direct access to all the camera functions and it is a basic tool to display images. It is recommended to use the Software Development Kit for a more flexible camera control. Due to the start-up time needed by the internal firmware to be fully working, the *VisualSPC³* should be started at least 15 seconds after having powered the SPC³. Failing to do so will generate an error by the software. Simply close and restart the software.

## *Installation*

Double-click the desired installer (32-bit or 64-bit) and follow the guided installation procedure. Please note that device driver from OpalKelly, provided on the SPC3 USB-Key, must be installed before connecting the camera to the PC.

## *Software Interface*

The *VisualSPC³* interface is composed a main window divided in several sections as showed in Figure 12, and a separate window for each active counter (see **Figure** 13). An additional window will also pop-up when FLIM mode is enabled (see ).

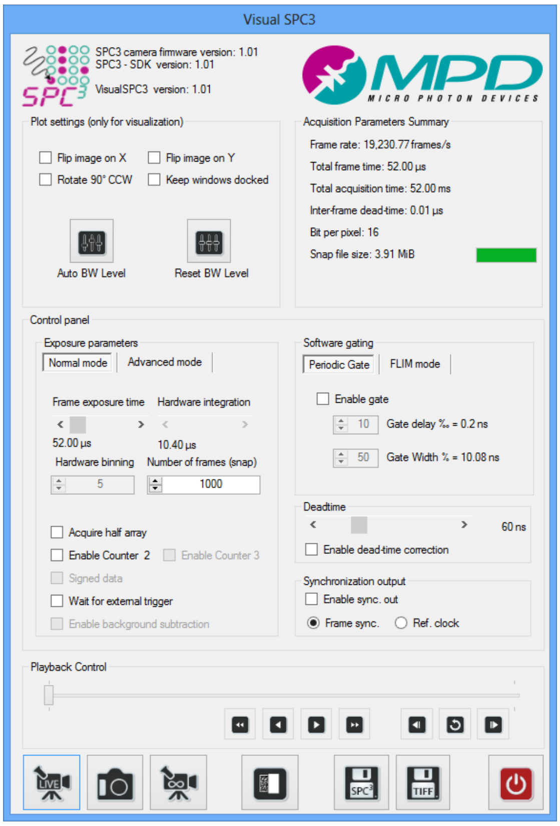



Figure 12. Screenshot of the main window of the graphical user interface.

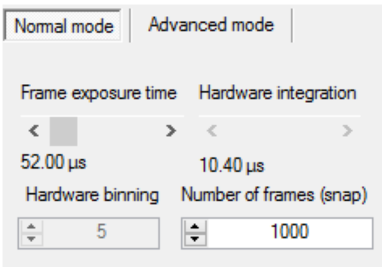
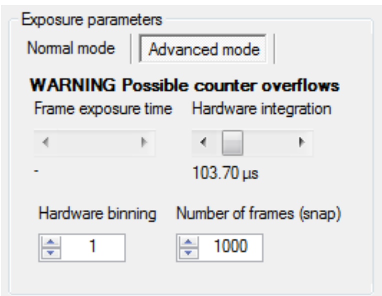
## *Main window: Control Panel*

This panel allows setting all the camera hardware parameters.

In the Exposure Parameters subsection it is possible to set the image integration parameters (*exposure time*, *number of frames* for Snap mode and *hardware binning*). Either *normal* or *advanced* modes are available. There are four numeric fields (see Table 3 for an example of settings), which can be either editable or automatically adjusted by the software depending of the selected mode, namely:

- ✓ *Frame exposure time (Normal only):* integration time for each frame required by the user. The possible values range between 10.40 µs and 2.40 ms. This value does not correspond to the *Hardware Integration Time* of the sensor. Longer exposure times are obtained by binning a variable number of frames.
- ✓ *Hardware Integration time:* physical integration time of the SPC$^3$ sensor. This value is fixed to the *Hardware Readout Time* in *normal mode*. In *advanced mode*, it can range from 10 ns to 655.25 µs.
- ✓ *Hardware binning:* number of frames of duration equal to *Hardware Integration Time* that are summed-up before being stored in the computer memory. In *Advanced* mode this field can be changed by the user, in *Normal* mode it is adjusted by the software depending on the chosen *Frame Exposure Time*
- ✓ *Number of frames*: number of frames that are acquired during a *Snap* event. This number ranges from 1 to 65534 for 16 bit images and to 131070 for 8 bit images.

**Table 3. Possible configurations of the Exposure Parameters subsection (Normal and Advanced mode)**

| | |
|---|---|
| *Normal Acquisition Control Box*<br> | The duration of a frame is obtained by binning a sequence of frames of shorter exposure times. In this example, a 52.00 µs *Frame exposure* is obtained by summing 5 frames of 10.40 µs. This acquisition is then repeated 1000 times and the data are transferred to the host computer. Practically, 5000 frames of 10.40 µs are acquired and binned by a factor of 5. This acquisition mode does not degrade the signal-to-noise ratio of the images because no read-out noise is present. |
| *Advanced Acquisition Control Box*<br> | The user has a full control on the frame acquisition.<br>In the example, each frame has an effective duration of 103.7 µs and no binning of the frames is performed (*Hardware binning* = 1). The acquisition is then repeated 1000 times and the data are transferred to the host computer. Frame exposures down to 10 ns can be obtained.<br><br>**WARNING** the integrated counters can overflow at long exposure times. |

In this subsection there is also a set of checkboxes to further customizing the acquisition. The full list and their explanation can be found in Table 4.

**Table 4. List of checkboxes in the Exposure Parameter subsection and their explanation**

| | |
|---|---|
| *Acquire half array* | Only the central 32x32 portion of the array is acquired. Useful to reduce required bandwidth. |
| *Enable Counter 2*<br>*Enable Counter 3* | Enable the extra in-pixel counters. When a counter is enabled a new Counter window pop-ups, when a counter is disabled the corresponding window is closed. |
| *Signed data* | When at least Counter 2 is enabled, this checkbox indicates if the data from Counter 2 and 3 has to be considered signed or not. Useful when these counters are employed in up/down mode. |
| *Wait for external trigger* | The camera waits for an external trigger on the TRIG IN input before starting the acquisition of an image sequence. |
| *Enable background subtraction* | The acquired dark frame is subtracted by the output image. A dark frame acquisition must have been previously performed (see the Control Buttons paragraph). |
| *Force 8bit mode* | Available only in *Advanced* mode. Data are truncated to 8bit. Useful to reduce required bandwidth. |

In the Software Gating subsection it is possible to configure the internal gate generation, which applies only to Counter 1. Two gated operation mode are possible, *Periodic Gate mode* and *FLIM mode*. In *Periodic Gate mode* a periodic signal is continuously applied to Counter 1, as explained in Table 5.

**Table 5. Periodic Gating section configuration explained.**

| | |
|---|---|
| Enable Gate | Enable/Disable the hardware gating |
| Delay | Change the delay of the gate signal respect to the internal reference clock. The delay value is expressed as a percentage of the reference clock period (20 ns). E.g. Delay = +10% → +2 ns delay |
| Width | Duration of the gate-on signal. This is expressed as a percentage of the internal reference clock period (20 ns). E.g. Width = 20% → 4 ns width. |

In *FLIM mode* the internal gate is automatically controlled in order to perform a gated FLIM acquisition with parameters explained in Table 6. See paragraph on FLIM operation for further details.

**Table 6. FLIM mode section configuration explained.**

| | |
|---|---|
| Enable FLIM mode | Enable/Disable FLIM mode. When FLIM mode is enabled an additional FLIM window pop-ups (see paragraph below). |
| Gate Width | Duration of the gate-on signal. This is expressed as a percentage of the internal reference clock period (20 ns). E.g. Width = 20% ➔ 4 ns width. |
| FLIM steps | Number of steps to be performed for each FLIM acquisition. |
| FLIM shift per step | Gate shift for each FLIM steps in thousandths of reference period. E.g. Step = 50‰ ➔ 1 ns step |
| FLIM Start position | Start position of the of the first FLIM step with respect to the internal reference clock. The delay value is expressed as a percentage of the reference clock period (20 ns). E.g. Delay = -10% ➔ -2 ns delay |

In the Deadtime subsection it is possible to adjust the hold-off time (in the range 50÷ 150 ns) and enable the embedded dead-time correction.

In the Synchronization Output subsection it is possible to enable the generation of voltage pulses on the SYNC OUT output. Two possible synchronization signals are available:

- ✓ *Frame sync*: a 50 ns pulse at the beginning of each frame
- ✓ *Ref*. Clock: the internal 50 MHz clock is replicated to this output.

## *Main Window: Plot settings and Playback Control*

The plot settings apply only to the images displayed on the screen. No changes are introduced to the data in the memory buffer. Therefore, these parameters do not affect the saved images. All this settings can be changed in real-time during display. The plot settings can be adjusted as explained in Table 7.

After a Snap has been acquired, it is possible to review the images by using the controls shown in Table 8. It is also possible to move along the data using the scrollbar. The current frame number and FLIM step (if in FLIM mode) are also shown.

**Table 7. Plot settings and control buttons.**

| | |
|---|---|
|  | Automatically sets the black and white levels of the image according to the present frame: the black level is assigned to the pixel with the minimum number of detected photons in the frame while the white level is assigned to the ones with the maximum count. This setting will remain applied also for the following frames. Since it is possible to have in the following frames pixels with values lower than black or higher than white, they will be respectively indicated in blue or red. |
|  | Resets black and white levels to default values: 0 ÷ 255 for 8-bit images, and 0 ÷ 65535 for 16-bit images. |
| *Flip X* | Flip the image along the X-axis. |
| *Flip Y* | Flip the image along the Y-axis. |
| *Rotate 90 °CW* | Rotate the image by 90° clockwise. |
| *Keep Windows Docked* | If selected, moving the main window will also move together all the other windows. |

**Table 8. Live viewer control buttons.**

| | |
|---|---|
|  | Go to the first acquired frame |
|  | Go to the previous frame |
|  | Go to the next frame |
|  | Go to the last acquired frame |
|  | Play the image sequence backward |
|  | Continuous rewind. When the last (first) acquired frame is displayed, it restarts from the first (last) frame |
|  | Play the image sequence forward |

## *Main Window: Control Buttons*

**Table 9. Camera control buttons for starting, recording and saving frame acquisitions.**

| | |
|---|---|
|  | Start the live-mode acquisition. Once the acquisition has been started, the button becomes a red box. Press it again to stop the acquisition. In FLIM mode the average counts over all FLIM steps is visualized. |
|  | Starts the acquisition of a sequence of frames (Snap mode). If no external trigger is requested, the acquisition starts immediately and the button becomes a red box. If an external trigger is required, the button turns first in an orange box, then in a red one only when the trigger is detected and the acquisition really starts. When the box is orange, a click on the button stops the waiting for a trigger. When the box is red, the user must wait for the end of the acquisition. |
|  | Starts the continuous acquisition of frames. If no external trigger is requested, the acquisition starts immediately and the button becomes a red box. If an external trigger is required, the button turns first in an orange box, then in a red one only when the trigger is detected and the acquisition really starts. For interrupting the waiting for the external trigger signal or the acquisition, once it has been started, press the button.<br>**WARNING**: Continuous mode acquisition may generate huge amount of data in short time, depending on acquisition settings. Check "Total saved data" in the *Acquisition Parameters Summary box* for the actual size of data saved on the HDD. |
|  | Performs the acquisition of a background frame. Once the light is prevented to reach the detector, only dark counts are measured. A dark frame is generated by averaging the *number of frames* acquired. This frame is then sent to the acquisition electronics to perform hardware background subtraction. The acquisition of the background frame must be performed using the same parameters that will be used for the actual measurement. Any change in the acquisition parameters, including the gate parameters and the dead-time correction settings, REQUIRES a new acquisition of the background frame. The background frame is not stored inside the camera and is lost when the camera is switched off. |
|   | Saves the acquired data by a Snap into a SPC3 format file or special SPC3 FLIM format. |
|  | Saves the data acquired by a Snap into a multipage TIFF file. The file is compliant with the OME-TIFF specification, which allows embedding in the file metadata all the acquisition parameters for the specific measurement, as well as FLIM mode parameters. OME-TIFF file can be opened with any image reader compatible with TIFF file, since metadata are saved into the Image Description tag in XML format. In order to decode OME-TIFF metadata, it is possible to use free OME-TIFF reader, such as OMERO or the Bio-Formats plugin for ImageJ. For more details see the OME-TIFF web site:<br>http://www.openmicroscopy.org/site/support/ome-model/ome-tiff/.<br>OME-TIFF metadata for FLIM mode acquisition include the *ModuloAlongT* tag, which allows the processing of FLIM data with dedicated FLIM software such as FLIMfit (see http://www.openmicroscopy.org/site/products/partner/flimfit). |
|  | Exits VisualSPC³ |

## *Main Window: Acquisition Parameters Summary*

In this section, the information on the acquisition, based on the actual parameters, is reported. The explanation of all the shown parameters is reported in Table 10.

**Table 10. Explanation of the parameters shown in the *Acquisition Parameters Summary* panel.**

| | |
|---|---|
| *Frame rate* | This is the actual frame rate of a *Snap* or *Continuous Acquisition* |
| *Total Frame Time* | This is the actual frame exposure time (i.e. the real *Hardware Integration Time*, multiplied by the number of internal sums) |
| *Total acquisition time* | This is the total time required to conclude the *Snap* acquisition of $N_{frames}$ |
| *Interframe dead-time* | This is the actual inter-frame dead-time. Normally it is < 10 ns long, but may increase in *Advanced* mode if very short *Hardware Integration Times* are selected. |
| *Bit per pixel* | Number of bit per pixel (8 or 16) |
| *Snap file size* | File dimension for a *Snap* acquisition with the current parameters |
| *Total saved data (only visible during Continuous Acquisition)* | This is the actual size of data so far saved to the disk. The number is updated in real-time after every write operation. |

## *Counter Window*

VisualSPC³ will show up to three windows (see **Figure** 13) showing the map of the accumulated counts in each counter. Window for Counter 1 is always visible, windows for Counter 2 and 3 will pop-up if selected in the *Exposure Parameters subsection* of the *Control Panel*. In addition to settings shown in Table 7, it is possible to manually adjust the black and white levels of the image and its contrast by using the controls on the color-bar at the left of the image. After a Live or Snap mode acquisition is stopped, it is possible to directly read the number of photons detected by each pixel by simply moving the mouse pointer over the image (available only if window size is set to the default value. Check “Keep windows docked” to revert to default size).

In FLIM mode only Counter 1 window is available. In Live mode the average counts over all FLIM steps are visualized, whereas in Snap mode counts for each individual FLIM step are shown. For the sake of clarity let’s consider a Snap of 10 FLIM acquisitions (camera in FLIM mode) and let’s suppose that each acquisition is composed by 50 steps (Gate Shifts, i.e. ‘Gate positions’): a total of 500 frames will be thus shown in Counter 1 window, following a "FLIM first, time second scheme". With such scheme, the frame sequence would follow this ordering: 1$^{st}$ gate shift of 1$^{st}$ FLIM measurement, 2$^{nd}$ gate shift of 1$^{st}$ FLIM measurement, ..., n$^{th}$ gate shift of 1$^{st}$ FLIM measurement, 1$^{st}$ gate shift of 2$^{nd}$ FLIM measurement, 2$^{nd}$ gate shift of 2$^{nd}$ FLIM measurement, ..., n$^{th}$ gate shift of 2$^{nd}$ FLIM measurement, etc.

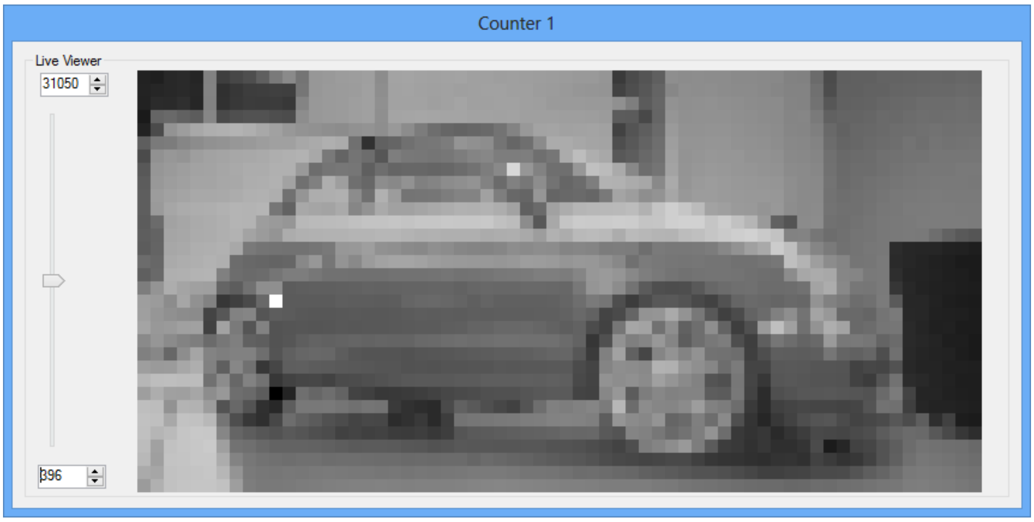



Figure 13. Screenshot of the Counter window.

## *FLIM Window*

When FLIM mode is enabled, an additional FLIM window pop-ups, as shown in . This graph represents the counted photons in each step (Gate Shift) of the current FLIM acquisition for a selected pixel, and it is thus the TCSPC histogram that reconstructs the waveform under investigation convoluted with the IRF of the system. The IRF is roughly a rectangular shape with a width equal to the Gate width. To change which pixel is visualized, simply click on the desired pixel in Counter 1 window. The graph is updated in real time in Live mode. In Snap mode it shows the entire FLIM waveform corresponding to the currently visualized FLIM acquisition (i.e. it will not change if, in Counter 1, a different FLIM step from the same FLIM acquisition is visualized ). It is possible to choose between linear and logarithmic scale for the Y axis. The *Accumulate* checkbox, if selected, will cause the histogram to accumulate counts among frames.

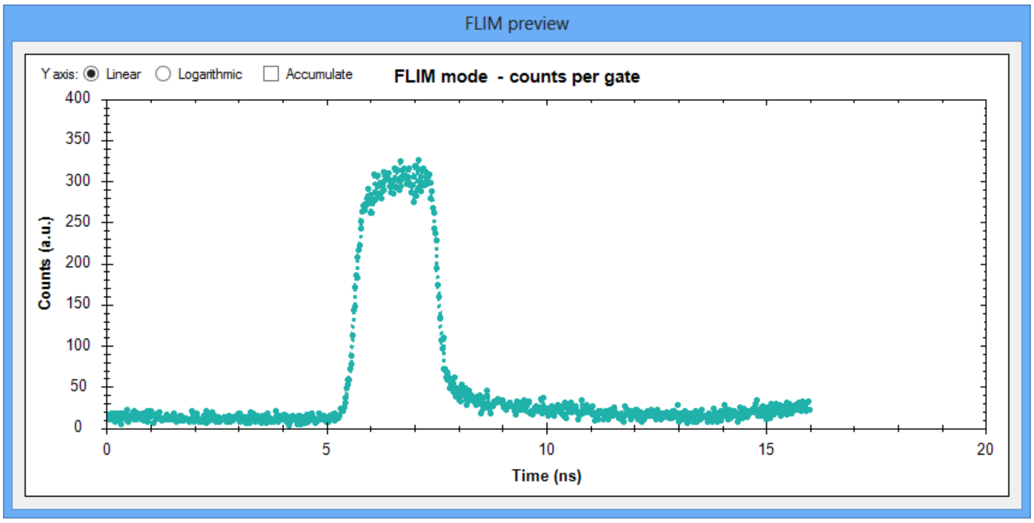



Figure 14. Screenshot of the FLIM window. In this case, the acquisition is the IRF of the system when Gate width is set to 2 ns, and 800 gate steps spanning 16ns are acquired.

# SPC3-SDK, File Formats and ImageJ Plugin

SPC$^3$ is provided with a Software Development Kit (SDK), composed by a shared library and few example projects. The SDK allows the user acquire data from the SPC$^3$ and to change operating parameter directly from their own application. It is available for Windows, Mac OS X and Linux operating systems, both in 32- and 64-bit versions. More information on available functions can be found in the related documentation included in the USB key.

Acquired data from Snap acquisitions can be saved both in OME-TIFF format and in a MPD proprietary binary file format. Data from Continuous acquisition can only be saved in MPD proprietary format.

OME-TIFF file could be opened with any image reader compatible with TIFF file, since metadata are saved into the Image Description tag in XML format. In order to decode OME-TIFF metadata, it is possible to use free OME-TIFF readers, such as OMERO or the Bio-Formats plugin for ImageJ. For more details see the OME-TIFF web site: http://www.openmicroscopy.org/site/support/ome-model/ome-tiff/.

Proprietary format files produced by SPC3 camera are based on the same binary structure, but different file extensions are used for differentiate among data. Photon counting acquisitions are stored in *.spc3* files whereas FLIM mode acquisitions are saved in *.spcf* files. In addition, each camera is provided with an *.spce* file containing measured photon detection efficiency. All these files contain binary data, composed by a header with acquisition metadata followed by raw image data which contain the 8/16 bit pixel values in row-major order. The byte order is little-endian for the 16 bit data. Raw data have the following meaning:

.spc3 files: data represents photon counts in each pixels during each Exposure Time, with acquisition parameters reported in the header. In case more counters are used, data are interlaced, i.e. the sequence of frames is the following: $1^{st}$ frame of $1^{st}$ counter, $1^{st}$ frame of $2^{nd}$ counter, $1^{st}$ frame of $3^{rd}$ counter, $2^{nd}$ frame of $1^{st}$ counter, etc.

.spcf files: data represents photon counts in each pixels during each Exposure Time, with acquisition parameters reported in the header. Images follow a "FLIM first, time second scheme", $1^{st}$ gate shift of $1^{st}$ FLIM measurement, $2^{nd}$ gate shift of $1^{st}$ FLIM measurement, ..., $n^{th}$ gate shift of $1^{st}$ FLIM measurement, $1^{st}$ gate shift of $2^{nd}$ FLIM measurement, $2^{nd}$ gate shift of $2^{nd}$ FLIM measurement, ..., $n^{th}$ gate shift of $2^{nd}$ FLIM measurement, etc.

.spce files: data represent measured Photon Detection Efficiency for each pixel. Values range from 0 to 10000, i.e. a 32.5% PDE will be expressed as 3250. Each frame represents a wavelength, whose range and step are reported in the header.

 *Micro Photon Devices S.r.l. – Italy*  

The header is composed by a signature of 8 bytes, and a metadata section of 32 bytes, as reported in Table 11. Note that multibyte fields are little-endian.

**Table 11. Header structure of SPC3 proprietary file formats .spc3, .spcf, .spce.**

| Byte offset | Number of bytes | Description |
|---|---|---|
| 0 | 8 | File signature: 0x4d5044ff03000000 |
| 8 | 1 | Number of rows |
| 9 | 1 | Number of colums |
| 10 | 1 | Bit per pixel |
| 11 | 1 | Counters in use |
| 12 | 2 | Hardware integration time (multiples of 10ns) |
| 14 | 2 | Summed frames |
| 16 | 1 | Dead time correction enabled |
| 17 | 1 | Internal gate duty-cycle (0-100%) |
| 18 | 2 | Holdoff time (ns) |
| 20 | 1 | Background subtraction enabled |
| 21 | 1 | Data for counters 1 and 2 are signed |
| 22 | 1 | FLIM enabled |
| 23 | 1 | FLIM shift % |
| 24 | 1 | FLIM steps |
| 25 | 4 | FLIM frame length (multiples of 10ns) |
| 29 | 2 | FLIM bin width (fs) |
| 31 | 1 | PDE measurement |
| 32 | 2 | Start wavelength (nm) |
| 34 | 2 | Stop wavelength (nm) |
| 36 | 2 | Step (nm) |
| 38 | 2 | Unused |

In order to easily visualize and elaborate data stored in the proprietary *.spc3, .spcf and .spce* file formats, a plugin for ImageJ software (http://rsb.info.nih.gov/ij/) is also provided. The plugin is compatible both with ImageJ (tested on v. 1.49q) and with Fiji (http://fiji.sc/Fiji), which is essentially a distribution of ImageJ together with several useful plugins.

In order to install the plugin follow this procedure:

- for *ImageJ* (tested on v. 1.49q), launch ImageJ and select the menu item *Plugins → Install…*, then follow the instructions and select the file *SPC3_Reader.java* provided in SPC3 USB key. The *"Save Plugin, Macro or Script..."* dialog appears with a list of your currently installed ImageJ plugins. Select a folder, such as the Input-Output folder, and save the *SPC3_Reader.java* file there. Do not change the name *SPC3_Reader.java*, as it follows ImageJ naming conventions and it is not seen by ImageJ if it does not contain an underscore.
- for *Fiji*, launch Fiji and select the menu item *Plugins → Install Plugin…*, then follow the instructions and select the file *SPC3_Reader.java* provided in SPC3 USB key. Restart Fiji.

In order to use the plugin select *SPC3_Reader* under the *Plugins* menu. It will be in the location you've previously chosen for ImageJ, or at the end of the menu for Fiji. An *"Open SPC3 file..."* dialog appears. Select the *.spc3, .spcf or .spce* file you wish to load. ImageJ will now ask you how to deal with signed data.

If data is 8bit, there are three options:

- Convert data to 32bit float
- Import unsigned data as is and convert signed data to 32bit float
- Import all data as is (this means ImageJ will interpret signed data as unsigned, e.g. the number -1, which in 8 bit binary is 11111111, will be shown as 255)

If data is 16bit, there are four options:

- Convert data to 32bit float
- Import unsigned data as is and convert signed data to 32bit float
- Import all data as is (this means ImageJ will interpret signed data as unsigned, e.g. the number -1, which in binary is 11111111, will be shown as 255)
- Import all data as is and use an ImageJ calibration function to map signed data (this means ImageJ will remap data to show signed values, e.g. the number -1, which in 16 bit binary is 1111111111111111 and will read as 65535 without remapping, will be properly shown as -1).

If the selected file is properly formatted and no error occurred, an image window for each SPC3 counter enabled opens. Frames can be skimmed using the controller at the bottom of each window. Selecting from the menu the command *Image→Show Info…* will show all camera parameters for the acquisition.

# System requirements

- High-speed USB 3.0 interface for full speed acquisition, compatible with USB 2.0
- Host computer (minimum requirements)
  - 2 GHz processor and 1 GB of RAM
  - SSD recommended for full speed continuous acquisition.
- Supported operating systems
  - VisualSPC$^3$
    - Microsoft Windows 7, 8, 32 or 64 bit versions
  - SDK and ImageJ Plugin
    - Microsoft Windows ,7, 8, 32 or 64 bit versions
    - Linux Ubuntu 12.04 LTS, CentOS 6.5, 6.6, 6.7 or compatible distributions, 32 or 64 bit versions. Different distributions should work, but were not tested.
    - Mac OS X 10.8 and above
- Tested ImageJ versions (different versions should work, but were not tested)
  - Standalone ImageJ: version 1.49q
  - Fiji distribution: version 2.0.0-rc-28/1.50b

# Copyright and disclaimer