SIXTH FRAMEWORK PROGRAMME PRIORITY:
IST FET Open

MEGAFRAME
Million Frame Per Second, Time-Correlated
Single Photon Camera

Deliverable D6.4
Laboratory fluorescence microscopy demonstrator

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<thead>
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<th>Author(s)</th>
<th>Change</th>
</tr>
</thead>
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<td>J. Arlt (UNIED)</td>
<td>First edited draft for internal circulation</td>
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<tr>
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<td>1.3</td>
<td>J. Arlt (UNIED)</td>
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Abstract
The present deliverable focuses on the Laboratory fluorescence microscopy experiments carried out as part of the MEGAFRAME project. This work has been predominantly focussed on time-domain fluorescence lifetime imaging microscopy (FLIM), with the addition of preliminary results on multi-point fluorescence correlation spectroscopy (FCS). Most of the reported work was obtained using Megaframe32 resin devices without microlenses mounted to them, but Section 9 contains some preliminary data for microlensed Megaframe32 arrays. The experiments highlight the different strategies that were explored to avoid the data-transfer bottleneck and utilise the available arrival time data efficiently, including on board lifetime estimation, on-board histogramming and on-board memory buffering. They also give some indication of the versatility of the MF32 detector, with demonstrations of fast lifetime estimation and TCSPC in wide-field fluorescence microscopy as well as TCSPC for scanning multi-focal confocal or multi-photon microscopy systems.

Given the delays in the availability of devices with improved sensitivity (i.e. microlenses mounted to recover some of the low fill-factor) most of the experiments presented here are proofs of principle, but one application project which is discussed in some detail (Section 7, Imaging of DNA microarrays) clearly demonstrates the scope of the device for biological and biomedical experiments.

Executive Summary
This report presents some preliminary results on **multi-point fluorescence correlation spectroscopy**, while being mainly focussed on different implementations and applications of **time-domain fluorescence lifetime imaging microscopy (FLIM)**.

In the first section some proof of concept results for **flexible multi-point fluorescence correlation spectroscopy (FCS)** are presented. In collaboration with the Laboratoire de Spectrométrie Physique (LSP) in Grenoble a setup was implemented that could generate 4 independently moveable laser foci on the sample which were then imaged onto 4 pixels of the Megaframe32 device. This made it possible to record FCS data in four locations simultaneously and first results indicate that performance comparable to existing techniques can be achieved.

As most of the wide-field fluorescence lifetime evaluation experiments have already been presented in previous deliverables, the first two FLIM experiments discussed in this report explore the use of Megaframe for **multi-spot scanning imaging setups**. This overcomes the limited spatial resolutions of the current device and somewhat alleviates the performance loss due to the small fill factor of the device at the expense of a more complex setup and slower speed.

- **First, multifocal multiphoton** FLIM experiments carried out in collaboration with Imperial College (London) are presented. High quality TCSPC FLIM data was acquired using **32 simultaneous excitation and acquisition channels**. This work makes full use of one line of detectors (32 elements) and builds up the TCSPC histograms on board of the imager. An optical scanning system allows for the reconstruction of larger images. Several FLIM images of samples such as pollen grains and plant cells (from Convallaria majalis) are presented. A sub-optimal optical setup lead however to long acquisition times (>1 hour) due to a low photon flux.
- Secondly, proof of concept results using single photon excitation with an array of 4 × 4 beams demonstrating basic confocal intensity imaging are shown. This mode actually takes advantage of the missing microlenses (just as the FCS implementation), dramatically improving the optical efficiency of the setup, and could easily be extended to confocal FLIM imaging using the now improved firmware.

The final part of the report is dedicated to fast wide-field TCSPC imaging.
- First, experiments performed by M. Gersbach from EPFL/TU Delft demonstrate high-speed and scanning-free TCSPC FLIM using Megaframe32. Wide-field FLIM acquisitions of a pollen grain sample with acquisition times as low as 69ms are shown. This high speed is achieved by building up arrival time histograms on board of the device.
- Secondly, the full potential to Megaframe32 was used at UNIED/COSMIC to image DNA micro-spot arrays at fluorophore concentrations comparable to those used in typical clinical assays. Such microarrays are important tools for biomolecular detection and are already widely used for gene expression profiling, disease screening, mutation and forensic analysis but also hold many promises for the future development of personalized drugs and point of care testing devices. Recently, fluorescence lifetime imaging (FLIM) has been reported as a powerful multidimensional technique that offers a number of benefits for microarray reading in term of sensitivity, specificity and multiplexing capabilities. By using the Megaframe detector to image these very weakly fluorescent samples a more than 100 times decrease in overall measurement time could be achieved, making this FLIM based analysis of micro-spot arrays a much more viable technique for high throughput screening.

Some very preliminary results obtained with microlens arrays mounted on Megaframe32 are also shown. On more than half of the pixels a concentration factor in excess of 10 was observed, reaching up to about 25. Although this spatially varying fill factor is not ideal, the device can produce relatively faithful images on half of the array. Wide-field TCSPC imaging of simple test samples shows arrival time histograms of good quality obtained at a more than 10 times higher speed.

**Reference Documents**

- D6.2 Evaluation report on the use of the phase 1 array for fluorescence detection (Interim version), 30/03/2009
- D6.2 Evaluation report on the use of the phase 1 array for fluorescence detection (Final version), 27/04/2010
- D3.2 and D5.1, Sensor Array, Phase 1 & Imaging system tape-out, Phase 1, FINAL Version – Experimental Results, 07/05/2010
- D4.2 Prototype Optical Concentrator, phase 1 - Supplement B: Measurements on the Mounted chip, 20/04/2010
- D6.3 PCB and FPGA with software for phase 2 array, 21/05/2010
## Conventions and Definitions

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Megaframe0”</td>
<td>Test chip containing SPAD test structures (6 rows of 17 SPADs each). Fabricated prior to project start.</td>
</tr>
<tr>
<td>“Megaframe1”</td>
<td>Test chip containing SPAD test structures (5 main structure types), as well as two analog 32-pixel arrays to investigate the performance of the analog read-out circuits. Fabricated early 2007.</td>
</tr>
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<td>“Megaframe1 respin”</td>
<td>Megaframe1 test chip resubmitted to the foundry early 2008 with a modified process flow, after extended pixel research activities to address dark count and spectral response improvements.</td>
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<tr>
<td>“Megaframe32 respin”</td>
<td>Low DCR version of Megaframe32, fabricated early 2009.</td>
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<tr>
<td>“Megaframe128”</td>
<td>160x128 array (originally 128x128), the main MEGAFRAME milestone, taped out June 2009, back from fab Sept 2009</td>
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<td>“Phase 1 chip”</td>
<td>The Megaframe32 device (32x32 array).</td>
</tr>
<tr>
<td>“Phase 2 chip”</td>
<td>The Megaframe128 device (160x128 array, originally 128x128).</td>
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## Glossary

<table>
<thead>
<tr>
<th>Term</th>
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<tr>
<td>APD</td>
<td>Avalanche Photo Diodes</td>
</tr>
<tr>
<td>CMM</td>
<td>Centre-of-Mass Method (Lifetime Estimation Algorithm)</td>
</tr>
<tr>
<td>DOE</td>
<td>Diffractive Optical Element</td>
</tr>
<tr>
<td>FLIM</td>
<td>Fluorescence Lifetime Imaging Microscopy</td>
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<td>FCS</td>
<td>Fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FPGA</td>
<td>Field Programmable Gate Array</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GUI</td>
<td>Graphical User Interface</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C (virus)</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human Cytomegalovirus</td>
</tr>
<tr>
<td>IEM</td>
<td>Integration for lifetime Extraction Method</td>
</tr>
<tr>
<td>IRF</td>
<td>Instrument Response Function</td>
</tr>
<tr>
<td>SLM</td>
<td>Spatial Light Modulator</td>
</tr>
<tr>
<td>N.A.</td>
<td>Numerical Aperture</td>
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<tr>
<td>SPAD</td>
<td>Single-Photon Avalanche Diode</td>
</tr>
<tr>
<td>TCSPC</td>
<td>Time-Correlated Single-Photon Counting</td>
</tr>
<tr>
<td>TDC</td>
<td>Time-to-Digital Converter</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total Internal Reflection</td>
</tr>
</tbody>
</table>
Table of Contents

Abstract ........................................................................................................................................... 3
Executive Summary ........................................................................................................................ 3
Reference Documents .................................................................................................................... 4
Conventions and Definitions ......................................................................................................... 5
Glossary........................................................................................................................................... 6
1 Introduction ............................................................................................................................ 8
2 Multi-point Fluorescence correlation spectroscopy ........................................................... 9
  2.1 Parallel FCS ...................................................................................................................... 9
  2.2 Preliminary experimental results ....................................................................................... 9
3 Overview of MEGAFRAME time-domain FLIM experiments ............................................ 10
4 Multiphoton multifocal FLIM ............................................................................................... 11
5 Scanning multi-focal confocal imaging ............................................................................. 15
6 Fast wide-field TCSPC-FLIM ............................................................................................... 17
7 Towards Real-time fluorescence lifetime imaging of DNA microarrays ......................... 22
  7.1 Introduction...................................................................................................................... 22
  7.2 Experimental details ........................................................................................................ 23
  7.3 Results and discussion .................................................................................................... 24
  7.4 Conclusion....................................................................................................................... 26
8 High-speed lifetime estimation using centre-of-mass method (CMM)............................ 27
9 Preliminary results obtained on microlensed Megaframe32 devices ............................. 27
10 Conclusions.......................................................................................................................... 30
11 Outlook.................................................................................................................................. 30
References..................................................................................................................................... 32
Appendix A: Details of the centre-of-mass (CMM) lifetime estimation method ...................... 34
1 Introduction

The design and characterization of SPAD arrays with complex integrated electronics for fluorescence lifetime imaging was detailed in previous deliverables (see Reference Documents) and some of the fluorescence microscopy demonstrations have already been covered in Deliverables D6.2 Interim & Final. This report aims to further prove the potential of this technology by showing some preliminary results for multi-point fluorescence correlation spectroscopy (FCS) as well as several examples of FLIM investigations we performed using MF32 SPAD detector arrays. An overview of the fluorescence microscopy experiments conducted within the current project, often in collaboration with external experts in the field, is summarised in Table 1. Only the experiments not reported in previous deliverables will be discussed here.

<table>
<thead>
<tr>
<th>Detector</th>
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<th>Type</th>
<th>Results</th>
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<td>Single SPAD</td>
<td>EPFL/TU Delft</td>
<td>TCSPC</td>
<td>Recording of high quality TCSPC data for a calcium indicator dye excited by multiphoton excitation</td>
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<td>MF32 (UNIED)</td>
<td>UNIED/COSMIC</td>
<td>wide-field FLIM</td>
<td>First demonstration of wide-field FLIM on bright samples using lifetime estimation (IEM), which yielded reasonable estimates despite the very high dark count rate</td>
<td>D6.2 Interim</td>
</tr>
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<td>MF32 respin (UNIED)</td>
<td>UNIED/COSMIC</td>
<td>wide-field FLIM using lifetime estimation (IEM)</td>
<td>Wide-field FLIM using lifetime estimation (IEM) on live biological samples. On bright samples such as microfluidic mixing, real-time FLIM was demonstrated.</td>
<td>[2] &amp; D6.2 Final</td>
</tr>
<tr>
<td>MF32 respin (EPFL)</td>
<td>EPFL/TU Delft with Imperial College</td>
<td>Multi-photon multifocal FLIM</td>
<td>Demonstration of scanning multi-photon multifocal TCSPC-FLIM using 32 pixels simultaneously.</td>
<td>D6.4</td>
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<tr>
<td>MF32 respin (EPFL)</td>
<td>EPFL/TU Delft</td>
<td>Fast wide-field TCSPC FLIM</td>
<td>Demonstration of fast wide-field TCSPC FLIM with on board histogramming</td>
<td>D6.4</td>
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<td>MF32 respin (UNIED)</td>
<td>UNIED/COSMIC &amp; UNIED with King’s College London</td>
<td>Confocal FLIM</td>
<td>Preliminary exploration of multi-focal scanning confocal imaging, including confocal FLIM</td>
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<td>UNIED/COSMIC with LSP in Grenoble</td>
<td>Flexible multipoint FCS</td>
<td>Demonstration of flexible multipoint FCS on 4 freely configurable pixels</td>
<td>D6.4</td>
</tr>
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<td>MF32 respin (UNIED)</td>
<td>UNIED/COSMIC</td>
<td>Fast wide-field TCSPC FLIM</td>
<td>Application of faster wide-field TIRF TCSPC FLIM to DNA microspot arrays</td>
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<td>Micro-lenses</td>
<td>UNIED/COSMIC</td>
<td>Intensity/TCSPC</td>
<td>Testing of microlensed MF32 device for TCSPC imaging</td>
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Table 1: Overview of fluorescence microscopy experiments conducted during the duration of the project.
2 Multi-point Fluorescence correlation spectroscopy

Fluorescence Correlation Spectroscopy (FCS) is a spectroscopy technique conceived in the early 1970s that uses confocal optics to detect fluorescence excited within the femto-litre volume of a tightly focussed laser beam [3]. By having a nano-Molar concentration of fluorescing molecules in the sample, the small volume ensures that an intensity fluctuation occurs as these molecules move in and out of the focused laser point [4]. By auto- or cross-correlating this intensity signal it is possible to obtain information about the sample, such as diffusion coefficients, molecule concentrations and flow rates [5].

2.1 Parallel FCS

Parallelism is the next logical step in terms of detection for FCS. Enabling multiple measurements to occur simultaneously will allow more complex analysis, as well as the ability to perform high throughput screening (HTS). A number of approaches have been studied;

- Multiple avalanche photo diodes (APDs) can be used together by coupling them to an array of fibre optic cables [6]. Due to the APDs size though, this technique does not scale well.
- High speed charge-coupled device (CCD) cameras can be used, but are both too slow to measure a micro-second time resolution and do not have single photon accuracy [7]
- Single photon avalanche diodes (SPADs) on a single CMOS substrate improve spatial and temporal resolution and have been demonstrated to work for FCS [8].

Optical set up is also important to create a matching array of parallel laser beams. The above methods use different approaches for optics including a total internal reflection (TIRF) laser [6], a spatial light modulator (SLM) [7] and a diffractive optical element (DOE) [8].

2.2 Preliminary experimental results

New hardware has been developed to support the Megaframe 32x32 sensor, replacing the existing development platform. As well as adding new functionality such as on-board bias generation, the new platform is smaller, more robust and less expensive than its predecessor. Firmware was implemented to assign each photon detection event a time stamp which is the time since the last event. These inter-photon-arrival-times for 4 freely selectable pixels provide the raw data which is sent to the PC and recorded. The correlation curves are then calculated from this raw data using algorithms based on papers by Wahl et al. [9] and Laurence et al. [10]. This is a standard form of data acquisition that was used as a proof of concept, but currently we are looking into a few other ideas for processing the data, including live correlation and calculation of concentrations/# molecules.

Preliminary experiments were carried out by David Tyndall (UNIED) in collaboration with the Laboratoire de Spectrométrie Physique (LSP) in Grenoble who wish to combine the sensor array with a novel approach to the optics. They want to combine the Megaframe sensor with an SLM which will pattern a custom array of laser beams onto the sample under observation [7]. This flexible approach saves the sample from being damaged, as would be the case with a static beam splitter such as a DOE.
Initial experiments for a single pixel confirmed that reliable results for a single focused laser beam can be achieved before moving on to incorporating the SLM for an increasing number of pixels. Subsequent experiments demonstrated the unique combination of both flexible illumination and flexible detection, where any 4 pixels on MF32 could be selected to perform the FCS experiments on. Such flexibility has never before been possible with single photon detection accuracy, with previous parallel experiments making use of detectors in a small grid, line [8] or cluster [6]. To underline this flexibility, the 4 channel experiment was carried out with a random selection of pixels within the 32x32 array, as shown in Figure 1(b). Diffusion coefficients extracted from these measurements were close to the literature values and those measured using a more conventional avalanche photo diode detection system on the same sample. However, alignment and focusing sensitivities meant that the number of molecules in the observed volume was larger than desired, hence lower G(0) values in FCS graphs compared to APD.

Figure 1: (a) FCS curves for a Sulfo-Rhodamine solution from 4 separate pixels in the sensor array with their least-square fits. (b) Inverted CCD image of SLM laser Illumination.

3 Overview of MEGAFRAME time-domain FLIM experiments

Laboratory fluorescence microscopy experiments carried out as part of the MEGAFRAME project have predominantly been focussed on time-domain fluorescence lifetime imaging microscopy (FLIM).

Early experiments conducted at the start of the Megaframe project showed the suitability of CMOS SPADs to acquire high quality fluorescence lifetimes using time-correlated single photon counting (TCSPC). Using a single SPAD linked to an external time-to-digital converter (TDC) the measured instrument response function (IRF) of 79 ps as well as the high lifetime accuracy for lifetimes as low as 100-200 ps prove the effectiveness of the chosen approach. Using the lifetime data collected of the high-affinity calcium indicator dye Oregon Green Bapta-1 (OGB-1) excited by a model-locked Ti:Sapphire laser operating at 800nm wavelength, a new triple-exponential decay model was introduced, allowing, as opposed to the conventional model, for the correct determination of the OGB-1-Ca$^{2+}$ dissociation constant. Details can be found in ref. [1].

First demonstrations of FLIM using the full Megaframe32 SPAD array were presented in the interim report for deliverable D6.2. The reduced dark count rate of the Megaframe32 respin device considerably increased the performance of the system, allowing for reliable real-time lifetime estimation based on the Integration for lifetime Extraction Method (IEM) [11] and improved..
performance on samples with low fluorescence, such as live biological cells. Details of these experiments can be found in the final version of the report for deliverable D6.2 and some of these results have also been published in ref. [2].

In the following we will present new experimental FLIM data obtained with the Megaframe32 respin device addressing most of the outstanding issues identified in deliverable D6.2. One of the major issues identified there was the low effective data bandwidth of the USB link from the imager to the PC. This meant that only a small fraction of detected photon events was actually transferred to the PC and therefore building TCSPC histograms on the PC was very inefficient. To overcome this issue, 3 separate approaches were implemented and tested. The first approach was to build up the histograms on the FPGA of the imager device, dramatically reducing the amount of data that needs to be transferred to the computer. This approach was used in the multi-photon multifocal FLIM experiments and the fast wide-field TCSPC-FLIM experiment conducted by the EPFL/TU Delft team presented in sections 4 and 6. The second approach was to record the TDC data into the on-board memory of the device and then compress the data and transfer it en-bloc to the PC. This approach was used by the UNIED/COSMIC team for the fast TCSPC-lifetime imaging of DNA microarrays presented in section 7. Thirdly, by implementing a more efficient data transfer protocol the available USB bandwidth was used much more effectively. Preliminary results obtained by the UNIED team showed continuous frame rates to the PC at 10kHz, i.e. 10 times faster than the 1000 fps achieved previously.

The second major outstanding issue was the poor photon detection efficiency of the SPAD array due to the low fill factor. The first Megaframe32 respin chips with microlenses mounted on them to increase the photon detection efficiency became available early in 2010, but poor uniformity of these early samples meant that they were not used in any of the FLIM demonstrations discussed so far. However, we will present some preliminary results on the last batch of microlensed Megaframe32 devices in section 9.

4 Multiphoton multifocal FLIM

In collaboration with S. Kumar, C. Dunsby and P. French at Imperial College, London, the possibility of integrating SPAD arrays with a multifocal multiphoton system for fast fluorescence lifetime imaging was investigated.

The use of multiphoton fluorescence microscopes has become widely popular as it offers distinct advantages over the single photon excitation scheme. With the fluorescence being excited solely at the focal spot, the 3D imaging capability is inherent to the multiphoton excitation technique. The precise localization of the fluorescence in a specific voxel is also beneficial in reducing out-of-focus photobleaching and photodamage [12]. Finally, the excitation light is less subjected to scattering thanks to its longer wavelength and thus, multiphoton excitation enables a higher sample penetration depth. However, the major drawback of this technique is its very low frame rate. In most cases, a single laser beam must be scanned across the sample to acquire a full image. The single acquisition channel and the time overhead of the scanning process results in very long acquisition times. The multifocal multiphoton [13] approach uses multiple laser beams and detection channels, thus, in theory, increasing the acquisition speed by a factor corresponding to the number of excitation/detection channels. Multiphoton Multifocal FLIM implementations have been reported using time-gated detectors [14-16], streak cameras [17] and a line of PMTs [18].
In theory, for a given resolution, the maximum frame rate of such a system is given by the photon rate, limited by pile-up and detector dead time effects, the scanning time and the data transfer and storage time. From [19] we know that a detection count rate inferior to 4% on each detector results in a pile-up distortion of the measured lifetime of less than 1%. Assuming that 1% error on the measured lifetime is acceptable, the maximum count per detector is in the order of 3.2 MHz for an 80 MHz laser repetition rate. For a detector dead time of 50 ns and a count rate of 3.2 MHz, the counting loss due to the detector dead time would amount to 16%. As a result, the maximum count rate per detector is \( \sim 2.7 \) MHz. Assuming that scanning and data operations are negligible in the overall frame acquisition time, the frame acquisition time can be approximated by:

\[
Frame \ acquisition \ time = \frac{R_x \cdot R_y \cdot P}{C_R \cdot D},
\]

where \( R_x \) and \( R_y \) are respectively the resolution in the x- and y-direction, \( P \) the desired number of photons to be acquired per pixel, \( C_R \) the count rate per pixel and \( D \) the number of simultaneously active detectors.

The electro-optical setup used to acquire multiphoton multifocal FLIM images is depicted in Figure 2. The femtosecond mode-locked Ti:Sapphire laser source (MaiTai Broadband, Spectra-Physics, USA) emits at 80 MHz repetition rate and a wavelength of 800 nm. A glass slit redirects a small fraction of the beam to a fast photodiode (Thorlabs, USA), the signal of which is amplified and sent through a variable delay line (Kentech Instruments Ltd., UK) and finally fed into the 32 x 32 SPAD array as reference clock for the TDCs. The remaining beam enters the TriMScope (LaVision Biotec, Germany) where it is multiplied into a user-defined number (1 to 64 beams with power of two increments) of beams arranged in line. The TriMScope contains pre-chirp compensation as well as a rotatable half-wave-plate/polarizer used for optical power attenuation. Finally the beam set is passed to the microscope (IX71 Olympus, Japan) and focused onto the sample. In the present case, 32 laser beams are used for excitation and a line of 32 detectors, out of the 32 x 32 array, detects the fluorescence emission. To acquire the FLIM images, the sample is displaced by a precision XYZ-stage.

**Figure 2: Electro-optical setup of the multiphoton multifocal FLIM experiments. Pr- Prism; L- lens; Pol-Polarizer, P-Pinholes; A- half-wave plates; IP- Image planes.**
The sample used for multiphoton multifocal FLIM acquisitions is a mixed pollen grain slide containing a mixture of pine, sunflower, cattail, alnus, evening primrose and lily pollen and a slice of lily flower (both Carolina Biological Supply Company, NC, USA). The pollen grains are stained with either Harris hematoxylin and fast green, or Harris hematoxylin and phloxine.

Figure 3: FLIM image of individual cells in a slice of lily flower. The resolution is 290 x 128 pixels (left) and 250 x 150 (right).
Figure 4: FLIM image of a pollen grain. Image resolution is 251 x 256 pixels.

Figure 3 and Figure 4 show the obtained FLIM images for both the pollen grain and the slice of lily flower. The vertical stripes are caused by four noisy pixels where the fluorescent signal is dominated by detector dark counts. It is believed that a more sophisticated fitting algorithm, with pixel-specific dark count compensation should enable extracting a lifetime for these pixels.

Unfortunately, the setup for the preliminary measurements shown here was not optimized and the total acquisition time was between 2-3 hours for the FLIM images shown. Several problems will have to be addressed to achieve a reasonable frame rate. Firstly, the acquisition software available at the time was not optimized and the histograms had to be transferred to the PC very frequently, resulting in a 30-40 % acquisition time overhead. The sample stage scan added 5 % to the total acquisition time while the detectors were recording the signal during 55-65 % of that time. The main source of losses was the optical system itself. The relative intensities of the fluorescence on the line of active pixels for 4, 8, 16 and 32 laser excitation beams are shown in Figure 5. One can notice significant crosstalk between the pixels resulting from a poor confinement of the fluorescent signal spots on the detector surface. With individual detectors being 50 μm apart and a detector active diameter of 8 μm, the fraction of photons impinging onto the active area of the detector is not exceeding a few percentage points. While microlenses could increase the fill factor of the detectors, more substantial changes to the setup will be required.

Nonetheless, the potential of the array was shown in these experiments and the successful acquisition of high resolution multiphoton multifocal FLIM images using a line of SPADs (from MF32) with integrated TDCs was shown.
5 Scanning multi-focal confocal imaging

The very small fill-factor of the Megaframe32 device is severely limiting its practical usability for wide-field imaging of sensitive samples such as fluorescently labelled living cells. However, we have explored the possibility of actually taking advantage of the small active detector area by using it as the confocal aperture of a multi-focal scanning setup. The basic idea is similar to the multi-focal FCS experiments were the excitation laser is focused in several spots in the sample plane which are then imaged onto individual pixels of the SPAD array.

For the initial experiments the confocal imaging was setup using a diffractive optical element in the beam path of the laser which created a $4 \times 4$ array of equally spaced spots. A magnifying beam relay was used to spatially filter out the remaining zeroth order light and image the laser foci into the sample plane. The fluorescence from this array of spots was then imaged onto Megaframe32 using an adjustable beam relay to demagnify the image such that a) the size of the spots is roughly the size of the photosensitive area of the SPADs and b) the spacing between spots corresponded to a multiple of the pixel separation. Using an SRL zoom lens (Sigma UC Zoom 28-105mm, 1:4-5.6) and a fixed television lens (16mm 1:1.4 Cosmicar television lens) the spacing of the spots could be adjusted to be exactly 3 pixels in both directions by using an overall demagnification of the relay of 3.57x. Intensity images where build up by scanning the sample over a regular range equivalent to the spot separation on the sample and recording photon count data at each location. From this data the $4 \times 4$ pixels were extracted and assembled into intensity images such as shown in Figure 6.

Figure 5: Relative fluorescence intensity for 4 (top left), 8 (top right), 14 (bottom left) and 28 (bottom right) laser beams.
Figure 6: Imaging two capillary tubes filled with Rhodamine 6G placed side by side at two different heights 200μm apart using a 4× objective. Top row shows the fluorescence wide-field image as well as the 16 focal points used for scanning. The second row is the reconstructed confocal image from an 8×8 scan obtained by manually scanning the sample.

At the time of these initial experiments the firmware did not allow for collection of TCSPC histograms on board or efficient collection using the memory controller and there was no option available to synchronise the data acquisition with the movement of the stage. Therefore only intensity images were collected, although it is in principle possible to collect multi-point confocal TCSPC data using the same optical implementation.

Although the initial experimental results look promising the optical sectioning achieved so far is still fairly poor. This is mainly due to the mismatch of fluorescence spot size compared to the active area in combination with the difficulties in achieving perfect alignment. The challenges faced here are similar to the problems encountered in mounting microlenses onto the device. The spacing of the spots has to be perfectly adjusted to the pixel spacing and its rotation adjusted to map the spots exactly to the SPAD array. However, in a properly designed optical system this can be tuned in situ with live feedback and might therefore easier to implement in practice.

Recently, a team from UNIED made a first visit to Simon Ameer-Beg’s Group at Kings College London to further pursue and extend this approach. Progress in terms of firmware, software and hardware achieved since the preliminary experiments should mean that multi-focal confocal FLIM imaging can be implemented readily. This work is ongoing and will represent an area of investigation beyond the current project.
6 Fast wide-field TCSPC-FLIM

In terms of fluorescence lifetime imaging the major advantage of the newly developed sensor is the availability of 1024 parallel FLIM acquisition channels capable of simultaneous acquisition. As opposed to conventional single detector FLIM systems, the present system can acquire a full image, albeit at low resolution, without the need of an optical scanning system. Thus significantly reducing the complexity of the optical setup and dramatically increasing acquisition speed. In this section, wide-field FLIM acquisitions using the Megaframe32 imager are presented. This experimental work was carried out by M. Gersbach at EPFL and TU Delft in collaboration with other group members, in particular, R. Trimananda wrote extensive software for data acquisition.

The imaging setup contains a pulsed laser source emitting short (<40 ps) light pulses at a frequency of 40 MHz, a wavelength of 405 nm and with an average power of 2 mW (Advanced Laser Diode Systems GmbH, Germany). The laser beam is then directed into a fluorescence microscope (BX51W, Olympus, Japan) where a dichroic beam splitter cube redirects the beam to the microscope objective (20 x, 0.45 NA, MPlanFL N, Olympus, Japan) and to the sample. The fluorescent light emitted by the sample is captured by the same objective and sent through the dichroic beam splitter, which filters out backscattered laser light, to the SPAD array. Time discrimination is performed on-chip using the in-pixel TDCs in reversed start-stop configuration. The stop signal is given by the lasers reference signal and the start by the SPADs. The fluorescence lifetime data is collected and stored as an individual histogram for each pixel on the motherboard. Finally, the collected data is read out to a personal computer through a USB link.

The IRF of the system was assessed by measuring the response of direct illumination from the laser onto the detector. The resulting curve is plotted in Figure 8.

Figure 7: Simplified schematic of the optical setup for wide-field FLIM. The detector was connected via a C-mount to the BX51W microscope (Olympus, Japan).
Figure 8: IRF of the wide-field FLIM system.

The sample used for the wide-field FLIM acquisitions is a mixed pollen grain slide (Carolina Biological Supply Company, NC, USA) containing a mixture of pine, sunflower, cattail, alnus, evening primrose and lily pollen. The pollen grains are stained with either Harris hematoxylin and fast green, or Harris hematoxylin and phloxine. The pollen grain studied in detail in this section is a bisaccate pollen grain of pine stained with Harris hematoxylin and phloxine. The two “sacs” or bladders facilitate wind dispersal of such grains and yield a significant lifetime contrast in the FLIM images (Figure 10 & Figure 11).

Figure 9: Intensity of the pine pollen grain used for wide-field fluorescence lifetime imaging. The highlighted pixel represents the position from which the fluorescence lifetime data in Figure 10 & Figure 11 was taken.

As the main advantage of the proposed setup lies in the high frame rate capability, the proposed measurements aim at showing the build-up of FLIM images for short acquisition times. Similarly to any other imaging system, the ultimate frame rate is limited by the number of photons impinging onto the detector and the measurement bandwidth of the system. The shot noise characteristic of single photon detection implies that a large number of photons must be measured to obtain reliable fluorescence decay information. In the present case the accuracy of the measured fluorescence decay was assessed by calculating the reduced chi-square ($\chi^2$) of the fitted double-exponential curve.
Figure 10: Fluorescence lifetime images and data and fluorescence lifetime data with the corresponding fitted curve from the pixel highlighted in Figure 9 for acquisition times of 69 ms (top left), 73 ms (top right), 130 ms (bottom left) and 254 ms (bottom right).
The reduced $\chi^2$ is defined as:

$$\chi^2 = \frac{1}{N} \sum_{i=1}^{N} \frac{(d_i - f_i)^2}{a_i},$$

where $N$ is the number of data points, $p$ the number of fit parameters, $d_i$ and $f_i$ respectively the data points and the fitted value.

Figure 10 and Figure 11 depict wide-field FLIM acquisitions with a resolution of 30 x 32 pixels of the above mentioned pine pollen grain. Fitting of the time-correlated fluorescence data was achieved with the help of the SPCImage software (Becker & Hickl GmbH, Germany). A double-exponential fit was found to best suit the data. The contrast of the images is given by the weighted average of the lifetime components:

$$\tau_{\text{mix}} = \sum_{i=1}^{N} \theta_i \tau_i \sum_{i=1}^{N} \alpha_i,$$

where $a_i$ and $\tau_i$ are respectively the partial amplitudes and the lifetime components. The same image was acquired multiple times with a different acquisition time to assess the optimal trade-off between frame rate and image quality. For each of the acquisitions, the fluorescence lifetime data and the corresponding double-exponential fit of a specific pixel (highlighted in Figure 9) is shown.

Figure 11: Fluorescence lifetime images and fluorescence lifetime data with the corresponding fitted curve from the pixel highlighted in Figure 9 for acquisition times of 454 ms (left) and 566 ms (right).

To show the reliability of the system and the good uniformity achieved when acquiring FLIM across the entire array, the $\chi^2$ maps for the shortest (69 ms) and longest (566 ms) acquisitions are
plotted in Figure 12. While it may not come as a surprise that the goodness-of-fit is improved for longer acquisition times, the good uniformity on the longer acquisition should be highlighted.

Figure 12: Goodness-of-fit ($\chi^2$) maps for acquisition times of 69 ms (left) and 466 ms (right).

We have shown that our SPAD-based device could reliably measure fluorescence lifetime images at frame rates unachievable with scanning systems. Ultimately, the acquisition speed limit is given by the data transfer rate and the time required to collect a sufficient number of photons, which in turn is dependent on detector efficiency and excitation conditions. Figure 13 shows the number of photons that were acquired on average for each pixel, including pixels where no fluorescent photons were impinging. For an acquisition time of 200 ms, which yielded a reasonably good fitting ($\chi^2 = 1.48$), the average photon number was only ~1150.

![Figure 13: Average number of photons per pixel and frame rate for various acquisition times. The values are extracted from the lifetime images in Figure 10 & Figure 11, plus additional acquisitions not shown.](image)

On average, the brightest pixel within the FLIM images shown in Figure 10 and Figure 11 detects a photon for every 240 excitations pulses, far from the photon detection limit given by pile-up considerations. Even for a very conservative ratio between excitation pulses and detected photons of only 1%, the acquisition time could be more than halved. If, as is often the case, a ratio of 4% is deemed acceptable, corresponding to 1 % error on the measure lifetime [19], the frame rate could be tenfold and well above video-rate. However, to achieve such photon flux on the relatively small pixel area, either a more powerful laser source or a fill-factor recovering scheme is required. In
many cases a higher excitation power is not desired as it increases photobleaching and photodamage. But as preliminary results (presented in section 9) with microlenses mounted on the SPAD array demonstrate a dramatic increase in acquisition speed can be achieved by the improved photon detection efficiency.

7 Towards Real-time fluorescence lifetime imaging of DNA microarrays

We report on the fast, near real time fluorescence lifetime imaging (FLIM) of DNA microarrays. The Megaframe32 respin imager was used to monitor the FLIM map of a two-plex DNA microarray excited by evanescent wave. The microarray was incubated with 10nM of complementary HCV and HCMV targets labelled with Alexa430 and Qdot525 respectively. At this concentration we were able to capture a 256 pixels FLIM map within less than 40s. These results are paving the way towards high throughput FLIM detection and fast lifetime multiplexing of DNA microarrays integrated onto compact commercial devices.

7.1 Introduction

DNA microarrays are important tools for biomolecular detection. Widely used for gene expression profiling, disease screening, mutation and forensic analysis, they also hold many promises for the future development of personalized drugs and point of care testing devices [20, 21].

In general terms, microarray technology exploits molecular recognition between a probe molecule attached to a substrate and a complementary target. The strength of the technique lies on its unique multiplexing capabilities, whereby a very large number of target molecules can be interrogated by a multitude of reporter probe spots printed on a single chip.

The read out of microarrays can be achieved using a variety of detection techniques [22], however, fluorescence is the most commonly used transduction method [23]. Recently, fluorescence lifetime imaging (FLIM) has been reported as a powerful multidimensional technique that offers number of benefits for microarray reading in term of sensitivity, specificity and multiplexing capabilities [24, 25]. Nonetheless, the relative complexity of the method, combined with bulky and expensive equipment has yet prevented its integration into microarray based biosensing devices.

FLIM can be performed using various methods either in the time or in the frequency domain. The respective advantages and disadvantages associated with different approaches have been reviewed in recent publications [26, 27]. The choice of the given technique is driven by specific applications. In the case of DNA microarray reading, time-correlated single-photon counting (TCSPC) based methods present some critical advantages over other techniques that include single photon sensitivity combined with unlimited dynamic range and high temporal resolution. The major draw back of TCSPC lies on its inherently low duty cycle and associated long data acquisition time, incompatible with real time monitoring and high-throughput screening.

But the MEGAFRAME imager is now enabling high parallelization of TCSPC detection in a compact and cost effective manner, allowing for the first time DNA microarrays readout with near real time capability.
7.2 Experimental details

We prepared a DNA microarray spotted with two different DNA probes and hybridized with their distinct complementary target conjugates. The detailed procedure of production and incubation has been reported in a previous publication [24]. In short, the microarray consisted of nine spots, incubated with a) hepatitis C virus (HCV) probe, b) human cytomegalovirus (HCMV) probe and c) a 1:1 molar ratio of HCV and HCMV probe. The array was hybridized with a solution of complementary target containing 10 nM of Alexa Fluor 430 (Alexa430) labelled HCV target and 10 nM biotinylated HCMV target, which was further incubated with quantum dot (QDs) streptavidin-conjugate solution, Qdot525. Qdot525 and Alexa430 were chosen for their overlapping absorption and emission spectra combined with a distinctly different excited state lifetime, approximately 4 and 25 ns respectively. Those properties permitted FLIM measurement using both a single excitation wavelength (415 nm) and a single read out channel (515 to 565 nm bandpass).

A schematic of the experimental setup is shown in Figure 1. The excitation source was a femtosecond Ti:Sapphire laser system (10W Verdi and Mira from Coherent, Glasgow, Scotland) producing pulses of 200 fs at 76 MHz. The output of the Mira was passed through a pulse picker, reducing the repetition rate to 4.75 MHz, and then frequency doubled to give an output at 415 nm. A fraction of the excitation beam was split off and sent onto a fast photodiode to obtain the stop trigger signal. The remaining portion of the excitation beam was directed to excite the DNA microarray by total internal reflection. In order to achieve this, a quartz prism (Cairn Research, Faversham, UK) was attached to the condenser of a Nikon TE300 inverted microscope and placed into contact with the undersurface of the DNA microarray using transmission immersion oil. The 2mW laser beam was directed below the critical angle and focused on to the DNA microarray, generating a local evanescent excitation of circa 1 mm² area. The resulting fluorescence was then collected with a 10× microscope objective (Plan Apo, N.A. 0.45, Nikon, Japan), filtered with an emission band pass filter (535/40 nm) and imaged onto the Megaframe imager. A de-magnifying
image relay ($f_1 = 50$ mm, $f_2 = 8.5$ mm) was used to match the excitation area to the field of view of the camera which has a small active area (1.6 mm squared).

Data presented here was acquired using the UNIED Megaframe32 respin chip, where the array is divided into two halves, the first half containing a fast TDC version (more accurate time resolution, shorter time range) and the second a slow one (less accurate time resolution, longer time range). For these experiments we used only the 32×16 pixels of the ‘fast’ TDC half, which has a time range of 50ns with 1024 bins of about 50ps resolution. The fluorescence intensity of the DNA microarrays combined with the low photon detection efficiency leads to fairly low photon count rate per pixel (peak rate of about 8kHz). To optimise the amount of useful data transferred to the PC, the frame rate of the imager was reduced to about 4 kHz by increasing the exposure time (see deliverable D6.2 for more details). This framerate is currently still too high to be continuously recorded, so blocks of 100000 frames with TDC codes were recorded on board (~26s total exposure time) and then transferred to the PC and combined into arrival-time histograms for each pixel (~12s in total). Custom software written in Labview (National Instruments, Texas) was used to remove the background in noisy pixels, provide an ‘instant’ lifetime estimate using the centre of mass method [28] or perform a least-square fit to a single exponential for each of the pixels.

### 7.3 Results and discussion

Figure 15 (a) shows the FLIM map of a DNA microarray spotted with HCV probe (bottom left), HCMV probe (top right) and a probe mixture containing 50% HCV and 50% HCMV (diagonal). For each spot, the individual emission decays of 16 pixels have been extracted. The lifetimes, calculated by a least squares fitting procedure of the decay tail to a single exponential, are reported in Figure 15 in the histograms b, c and d, which are presented with the corresponding Gaussian distributions. The HCMV and HCV-HCMV mixture displayed lifetimes centred on 4.65 ns, 24 ns

![Figure 15: (a) FLIM map of DNA microarray spotted with HCV probe (bottom left), HCMV probe (top right) and a probe mixture containing 50% HCV and 50% HCMV (diagonal). The array was hybridized with a solution containing 10nM of Alexa430 labelled HCV complementary target and 10nM of Qdot525 labelled HCMV complementary target. The fluorescence lifetime of the three spot categories were extracted for 16 pixels and are presented in histograms (b), (c) and (d) respectively.](image-url)
and 13.5 ns, matching the reported emission decay values for Alexa430 and Qdot525 [24]. Alexa labelled HCV probe spots presented a narrow lifetime distribution, while the Qdot525 labelled HCMV and mixture probe spots were less homogeneous. The different distribution spreads observed reflect the relative complexity of the emission decay(s) present in the three categories of probe spots. In the case of HCV, Alexa430 possess a simple, single exponential decay component, which translates into a narrow distribution of lifetimes with a FWHM value of 0.07ns. Conversely, the dynamic of the QDs excited state follows complex mechanisms, resulting in a multiexponential emission decays with up to 5 components [29]. In that case, the FWHM reach 0.9ns. The emission decay inhomogeneity increases further when both Alexa430 and Qdot525 are mixed together leading to a FWHM value of 1.4ns.

![Decay histograms for representative pixels for microspots labelled with Alexa, a mix of Alexa & QDots as well as QDots.](image)

Figure 16: Decay histograms for representative pixels for microspots labelled with Alexa, a mix of Alexa & QDots as well as QDots.

The 256 pixel FLIM map presented in Figure 15 (a) (corresponding to a quarter of the total pixels on Megaframe32) was obtained within 40sec. Of this time the sample is exposed to laser light for only 26 sec, the remainder is taken up by data transfer, histogram calculation and lifetime estimation.

Figure 16 shows decays for an individual pixel representative of each of the 3 different spots.
Assessment of different technologies is difficult as they often involve very different principles of operation, detector size, resolution and illumination. Compared with our recently published results measured on a similar sample and obtained with a state of the art TCSPC quadrant anode detector [24], the Megaframe32 acquisition time was about 200 times faster. With the quadrant anode detector it took about 30 min to collect about 500000 per microarray spot (imaging 9 to 12 of them simultaneously), whereas Megaframe could acquire 1.5M counts per spot in only 26s. This performance was achieved without microlenses mounted on the chip, so there is scope for the acquisition time and sensitivity to be further enhanced. Firstly, the light collection efficiency on each pixel is currently limited by the small SPAD active area (7 μm diameter) on a pixel pitch of 50μm × 50μm mostly taken up by its timing circuit, leading to a fill factor of only 2% [30]. Addition of microlenses is expected to enhance light collection by at least 10-fold [31], therefore increasing either the sensitivity or the lifetime update rate of the present device. Secondly, acquisition speed could also be increased by optimizing data transfer rates to the PC (latest camera board/firmware version can achieve continuous transfer to PC at about 10000fps) and/or by replacing the software based lifetime determination by an on-board method. The potential of this approach has already been successfully demonstrated by monitoring real time mixing of dye solutions in microfluidics using high-speed integration for extraction method (IEM) algorithms (see deliverable D6.2 (final version) and ref. [2]). For the low photon count rates achievable from the microarray samples the centre of mass lifetime estimation method might however be more appropriate [28].

Finally, the production of the larger Megaframe128 chip with up to 160 × 128 pixels will allow arrays of 256 spots to be measured in a single detection event, opening up a true opportunity for high throughput FLIM detection.

The microlenses should address one of the major drawbacks of the current device. To achieve the considerable 200 times speed increase, the total observed count rate from the sample has to be increased, which means that it has to be illuminated with much higher laser powers than for the TCSPC quadrant anode detector. As the detection efficiency of Megaframe without microlenses is currently almost 40 times lower, laser power on the sample has to be increased by almost four orders of magnitude, leading to a noticeable deterioration of the sample (particularly the Alexa dye) throughout the course of an afternoon of experiments. Increasing the detection efficiency would reduce the total sample exposure proportionally and therefore mostly alleviate this problem.

7.4 Conclusion

In summary, we have demonstrated that the Megaframe32 camera enables fast, near ‘real-time’ fluorescence lifetime imaging and multiplexing of DNA microarray using a SPAD array camera built in an economical and compact CMOS technology. Future upgrading of the SPAD imager will allow for faster and more sensitive detection to be reached on a larger sensor area. Such improvements will permit the high throughput FLIM of DNA microarrays and open the possibility to integrate FLIM transduction on biosensing and point of care testing devices.
8 High-speed lifetime estimation using centre-of-mass method (CMM)

To achieve real-time fluorescence lifetime imaging on dim samples we also explored the on board implementation of an alternative, more photon efficient lifetime estimation algorithm. Details of this fairly well established algorithm based on the centre-of-mass of the decay curve are discussed in appendix A, together with a comparison to other estimation methods. There, the merits of the CMM in terms of photon efficiency, lifetime resolvability, and on-chip feasibility are clearly demonstrated (see also ref [28]). This might make it the most suitable algorithm for imaging dim samples, although the efficiency advantage might be somewhat cancelled by its greater sensitivity to noise.

CMM has been implemented in firmware and initial test experiments using microfluidics have been performed, achieving very high lifetime update rates. This works well in certain experimental conditions (Figure 17) but in general the current implementation suffers the same problems as encountered with the IEM firmware implementation. Currently a lifetime estimate is returned even for very low photon counts, meaning that the displayed lifetime estimates quickly loose their likeness to the photon count image. This problem is in the process of being addressed by introducing a photon count threshold.

![Figure 17: Three snapshots from the graphical user interface of real-time CMM lifetime estimation on a 9μm diameter fluorescent bead in a microfluidic flow channel (images about 0.1sec apart).](image)

Software implementations in Matlab and Labview clearly show that this will then provide a fast, useful lifetime estimation (see Figure 15 (a) which is based on CMM estimates, as well as Figure 19). The recently implemented more efficient use of the USB bandwidth implies that full TCSPC data for dim samples can be transferred to the PC almost without any loss, meaning that software CMM works almost as efficiently as a firmware implementation. However, on-board lifetime estimation will be highly relevant once microlensed arrays and/or the much larger Megaframe128 device will be available as the number of photon events will once again exceed the available data transfer bandwidth.

9 Preliminary results obtained on microlensed Megaframe32 devices

At the time of writing this report the first partially successful chips with microlenses mounted just became available for lab testing. The most promising of these chips (PV_21) achieves concentration factors in excess of 10 in a reasonably uniform area covering about one half of the chip (see Deliverable 4.2, Supplement B). As can be seen from Figure 18, structures in the top half of the intensity image can be clearly recognized despite the residual variation in concentration factor in this part. But the rapid drop-off in concentration factor towards the bottom limits the useful area for imaging to the top half. Somewhat inconveniently, this area covers both halves of TDC ranges
with the highest concentration factors being achieved in the fast TDC half (marked in red on the figure).

Figure 18: Intensity image of a calibration scale using the micro-lensed chip PV_21. The field of view is illuminated evenly, the background should therefore appear uniform.

To benchmark the chip performance for wide-field TCSPC imaging, a test sample of fluorescent beads (Sigma-Aldrich, L4655, 1μm yellow-green fluorescent Latex beads) suspended in an 1mM aqueous solution of rhodamine B was imaged under very low laser illumination. Using only the fast TDC half of Megaframe32, arrival time data for (almost) the same field of view was collected using a chip without microlenses and PV_21 (Figure 19). The overall exposure time for PV_21 could be reduced by a factor of 16 compared to the chip without microlenses, while still giving a

Figure 19: Photon count image and CMM lifetime estimates based on wide-field TCSPC data of 1μm fluorescent beads in aqueous rhodamine B solution as well as decay histograms from representative pixels. a) using chip without microlenses b) chip with microlenses (plotted using the same scales).
similar number of counts in the decay histograms. The fluorescent beads show a bi-exponential decay with a much longer average lifetime than the rhodamine B and can thus easily be distinguished using a simple centre-of-mass estimation.

As expected, the microlenses increased the number of counts without affecting the overall shape of the arrival time histogram. Lifetimes extracted from the TCSPC histograms stays unchanged within the errors but the acquisition time required to achieve the same number of counts is reduced by the concentration factor. The very first tests were performed with very bright samples and it was noted that the camera would more easily ‘crash’, presumably due to over-exposure. However, subsequent testing with more realistic light levels, i.e. samples with much lower fluorescence signal, confirmed that this is not a limitation for bio-imaging applications.
10 Conclusions

In this document we have presented several laboratory microscopy experiments highlighting the potential of the Megaframe imager for fluorescence microscopy applications. By leveraging the high scalability and integration capability of the devices we have demonstrated preliminary results for flexible multi-point fluorescence correlation spectroscopy as well as a range of fluorescence lifetime imaging experiments.

Firstly, a 2-photon implementation with 32 excitation beams and the corresponding number of detection channels was presented. While severe improvements in the optical setup are still required, the potential of this technology in terms of acquisition speed is well established. Similarly, a multi-focal scanning confocal implementation was demonstrated.

Secondly, fast wide-field FLIM using time-correlated single photon counting was demonstrated, to the best of our knowledge, for the first time with such a large detector array. Whereas conventional TCSPC systems in most cases record images in minutes, our parallel acquisition scheme allowed for acquisition times well below one second.

As a final experiment the imager was used on DNA microarrays with low fluorophore concentrations representative of typical samples used in biological research or biomedical screening assays. Even without microlenses mounted Megaframe was able to speed up TCSPC FLIM imaging by a factor of 200 compared to previous experiments done on similar samples, paving the way towards real time imaging.

The one major weakness of the Megaframe32 detector limiting its performance in all reported wide-field experiments is its very low photon detection efficiency due to the small fill factor. Microlensed chips which achieve high concentration factors over a large area of the array only became available at the very end of the project, but preliminary results on these chips demonstrated that concentration factors far in excess of 10 were achieved in large parts of the array. This can either speed up image acquisition by the same factor and/or reduce the excitation laser intensity.

11 Outlook

The fluorescence microscopy experiments using the various SPAD arrays designed and fabricated throughout this project depended on the availability of such working devices for lab testing. As such, their advancement was affected by the speed of the progress, which was slower than initially hoped for, in producing such devices/systems working to full specifications. This implied that most of the lab evaluations performed so far, including most of the ones presented in this final deliverable report, remained on the level of ‘proofs-of-principle’ with some aspects of their evaluation still being far from complete. However, these demonstrations show great promise and clearly demonstrate the versatility of the device within the field of fluorescence and luminescence microscopy. The application project on imaging of DNA microarrays clearly demonstrates that even without microlenses Megaframe32 can outperform state of the art instruments considerably in terms of acquisition speed. This will enable ‘real-time’ wide-field TCSPC imaging, for example using TIRF illumination, on a wide range of biological and biomedical samples.

Maybe even more importantly for the overall success of the project the lab demonstrations provided valuable feedback on the performance and usability of the device. Although some of the dramatic improvements encountered during the course of the lab testing were due to better hardware (most notably the much lower dark count rates in the respin array), possibly much more
progress in usability has been achieved by steady improvements in firmware and data handling. This would not have been possible without the constant feedback from simple lab demonstration experiments. The feedback also was fed straight into the design of the larger Megaframe128 array which will hopefully make the lab testing of these new devices a much smoother and quicker process.

Obviously there are still many outstanding issues regarding fluorescence microscopy applications using the Megaframe SPAD arrays, which are subject to intense ongoing efforts that will continue far beyond the official end of the project. In many of the cases presented in this report the instrumentation aspects have so far not been fully addressed and the first promising microlensed arrays are only just becoming available for testing with ‘real’ samples. Other aspects of Megaframe relevant for fluorescence microscopy remain completely unexplored so far, such as its ability to image at very high frame rate at low light levels or to work in time gated mode. This will also be subject of future investigations.

The availability of the larger 160 × 128 array will address the low spatial resolution of the current device and therefore greatly increase its appeal to the biological and biomedical community. With this resolution it will be possible to acquire images directly comparable to the ‘standard’ resolution of most scanning FLIM systems.

As the DNA micro-spot array experiments demonstrate, the Megaframe32 device even without microlenses is already in a state where it is competitive with existing state-of-the art techniques. Megaframe128 with microlenses will clearly outperform alternative techniques in certain aspects of operation. Several world leading biological research groups using fluorescence microscopy techniques have contacted the partners and currently several scenarios of biology driven projects are actively explored.
References

Appendix A: Details of the centre-of-mass (CMM) lifetime estimation method

In the following we assume that a fluorophore has a single exponential emission behaviour. The single-exponential assumption allows a proper comparison of various fitting algorithms. Moreover, a single-exponential decay model is still useful to contrast different types of fluorophores. For diagnostic applications, obtaining lifetime contrast is probably more important than calculating the absolute values of lifetimes.

For an object with a continuous distribution of mass density \( f(r) \) and total mass \( M_T \), its centre of mass is defined as

\[
\text{CM} = \frac{\int r f(r) \, dV}{\int f(r) \, dV} = \frac{\int r f(r) \, dV}{M_T}. \tag{1}
\]

For a mass density of a single-exponential function \( f(t) = A \exp(-t/\tau) \) in the range \( 0 \leq t \leq T \), we have

\[
\begin{align*}
\int_0^T t f(t) \, dt &= \int_0^T A t \exp \left( -t/\tau \right) \, dt \\
&= A \tau^2 \left( 1 - e^{-T/\tau} \right) - A \tau T e^{-T/\tau} = \tau \int_0^T f(t) \, dt - A \tau T e^{-T/\tau}, \\
\Rightarrow \text{CM} &= \frac{\int_0^T t f(t) \, dt}{\int_0^T f(t) \, dt} = \tau - \frac{T e^{-T/\tau}}{1 - e^{-T/\tau}}. \tag{2}
\end{align*}
\]

As \( T > 7\tau \), the centre of mass lies at the position with a distance of \( \tau \) from the origin as Fig. 19(a) shows. If \( f(t) \) represents a fluorescence histogram, the denominator of Eq. (2) will be the total photon count, while the numerator is the sum of temporal information of total photon events. To implement Eq. (2) in hardware, denoted as CMM for simplicity, we need to quantize the temporal information by dividing the measurement window into \( M \) time bins (bin width of \( h \)), as shown in Fig. 19(b), using TDCs in the photon counting module. If the measurement window is comparable to the lifetime, a simple recursive program can be implemented on PC or a look-up table can be implemented on FPGA to calculate a more accurate lifetime using Eq. (2).
With a in-pixel TDC (bin width of $h$), the lifetime can be estimated through the temporal information of each photon event as

$$\tau = \left( \sum_{j=0}^{M-1} jN_j \right) \frac{1}{N_c} + \frac{1}{2} h, \quad (3)$$

where $N_j$ is the number of recorded counts in the $j$th time bin ($j = 0, 1, \ldots, M-1$), and $N_c = A\tau \left[ 1 - \exp(-Mh/\tau) \right]$ is the total effective signal count.

The precision and accuracy equations for Eq. (2) can be easily derived as

$$\frac{\Delta \tau_{CMM}}{\tau_{CMM}} = \frac{h}{\tau} G(x) - 1, \quad (4)$$

$$G(x) = \frac{1 + x - (2M + 1)x^M + (2M - 1)x^{M+1}}{2(1-x)(1-x^M)}, \quad (5)$$

$$\frac{\sigma \tau_{CMM}}{\tau_{CMM}} = \frac{h}{\tau \sqrt{N_c}} \frac{\sqrt{x - M^2x^M + (2M^2 - 2)x^{M+1} - M^2x^{M+2} + x^{2M+1}}}{(1-x)(1-x^M)}, \quad (6)$$
where \( x = \exp(-h/\tau) \). If the background count is considered, a DC value of \( C_0 \) obtained by averaging the counts of several bins on the flat response can be included in Eq. (3) to obtain a more accurate lifetime time. Suppose we have a white background noise response and we can therefore obtain the background count as \( N_b = MC_0 \) from Eq. (3) and by subtracting \( C_0 \) from the count in each bin, we have

\[
\tau_{CMM_{corr}} = \frac{\sum_{j=0}^{M-1} \Delta t_j (N_j - C_0)}{N_{total} - MC_0} = \frac{\sum_{j=0}^{M-1} \left(j + \frac{1}{2}\right) N_j - \frac{MN_h}{2}}{N_c}.
\]

Eq. (3) can be implemented on FPGA as shown in Fig. 20.

![FPGA implementation of CMM](image)

Figure 20: FPGA implementation of CMM.

From the discussion above, in the optimal window or lifetime resolving range for CMM, we have

\[
\tau / \sigma \tau = 2^{L/2} \quad \text{or} \quad SNR \cong 3L \quad (dB).
\]

This is a very convenient formula for end-users. By selecting a proper \( L \) via the graphical user interface (GUI), one can easily set the accuracy of images. For example, if a precision of \( SNR = 30dB \) is required for the system, the total count within the measurement window is \( 2^{SNR/3} = 1024 \). Figure 21 shows inverse precision curves versus total count for 800-bin CMM and 2-gate RLD (2\( \omega_g = 800h = 1\tau \) or 4\( \tau \)) with Monte Carlo simulations for different lifetimes. CMM displays its uniform performance and higher photon counting efficiency over a wide range of lifetimes. Therefore, CMM is suitable for low light detection. A comparison summary for the CMM, IEM, RLD, and the MLE algorithms is provided in Table I. It clearly shows the merits of the CMM in terms of photon efficiency, lifetime resolvability, and on-chip feasibility.
**TABLE I**

<table>
<thead>
<tr>
<th>Method</th>
<th>$F_{\text{MIN}}$</th>
<th>$F &lt; 4$ Resolvability</th>
<th>On-Chip Feasibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard RLD-2</td>
<td>1.5 @2.5</td>
<td>0.08 $&lt; \tau/MW &lt; 1$</td>
<td>Yes/Look-Up Table$^b$</td>
</tr>
<tr>
<td>IEM w/o Calibration</td>
<td>1.6 @0.67$^a$</td>
<td>0.06 $&lt; \tau/MW &lt; 0.7$</td>
<td>Yes</td>
</tr>
<tr>
<td>IEM with Calibration</td>
<td>1.2 @1.67</td>
<td>0.03 $&lt; \tau/MW &lt; 0.7$</td>
<td>Yes</td>
</tr>
<tr>
<td>MLE $M = 1024$</td>
<td>1.0 @[0.01–0.5]</td>
<td>1e-4 $&lt; \tau/MW &lt; 1.6$</td>
<td>No</td>
</tr>
<tr>
<td>CMM $M = 1024$</td>
<td>1.0 @[0.01–0.1]</td>
<td>3e-3 $&lt; \tau/MW &lt; 0.15$</td>
<td>Yes</td>
</tr>
<tr>
<td>CMM $M = 4096$</td>
<td>1.0 @[0.003–0.08]</td>
<td>7e-4 $&lt; \tau/MW &lt; 0.15$</td>
<td>Yes</td>
</tr>
</tbody>
</table>

$^a$The optimal $h$ of IEM is independent of $M$. $^b$On a small detector array.