Fluorescence Lifetime Imaging based on CMOS Single Photon Avalanche Diode Technology

Marek Gersbach

Prof. Edoardo Charbon

AQUA – Quantum Architecture Group
Outline

- Motivation and Objectives
  - Introduction to FLIM
  - Fluorescence
  - Data acquisition
- Detector
- Preliminary Results
- Outlook and future work
Motivation

- Show that SPAD detectors enable better temporal resolution and frame rates for FLIM imaging
- Gain new knowledge on neuronal activity through an improved FLIM imaging capability
Quantitative Bio-Imaging

- Detection of potential variations
  → Across the membrane
  → Across the cell
- Detection of auxiliary processes in neuronal communication
- Measure ionic concentrations such as Calcium concentration

[Zuschratter 2005]
Objectives

Short term:

- Prove the feasibility and utility of using SPAD detectors for FLIM imaging
- Assess the temporal resolution that can be achieved

Long term:

- Build a setup for FLIM imaging of deep neurons
- Design SPAD detectors specifically for this application
A specific fluorophore is characterized by its emission spectrum as well as by its unique lifetime.

Fluorescence lifetime is defined as the time in which the fluorescence intensity decays to 1/e of the initial intensity.

Fluorescence lifetime is influenced by changes in the cellular environment, such as pH, ion concentration or FRET.

[Agronskaia et al. 2004]
The laser emits a pulse
The excited sample emits photons
The detector counts the incoming photons
A histogram is computed
Fluorescence

- An incoming photon of the proper wavelength is absorbed by the fluorophore.
- The fluorophore relaxes to a lower vibrational state.
- A new photon of slightly lower energy is re-emitted.
Two-photon Fluorescence

- Two photons are simultaneously absorbed by the fluorophore.
- Fluorescence is limited spatially to the focal region.
- Photobleaching and photodamage is greatly reduced.

[www.phys.ntu.edu.tw/biophys/English/ETPM.htm]
Laser

- Tuneable femtosecond laser
- Pulsewidth less than 100 fs
- Tuneable from 710 to 930 nm
- Mode-locked at 80 MHz
- Designed for multiphoton applications

[www.newport.com]
Data acquisition

- The emission of the laser pulse is detected and acts as “start” for the Time-to-Digital-Converter (TDC)
- The laser pulse is cut off by a filter
- The detector sends a “stop” signal when a photon is detected
- The Time-To-Digital-Converter measures the time between the emission of the laser pulse and the detection of the fluorescent light
Histogram acquisition

- Frequency of the laser is 80 Mhz
- Lifetime decay is in the order of a few nanoseconds
→ Extremely rapid detectors are needed
Motivation and Objectives
- Introduction to FLIM
- Fluorescence
- Data acquisition

Detector

Preliminary Results

Outlook and future work
SPAD Detector

- Very high sensitivity: 
  Photon detection probability of up to ~25%
- Very fast: up to 25 million hits per second with a jitter of a few tens of ps.
- Can be produced in arrays

[C. Niclass et al. 2005]
SPAD: Operating principle

- Diode is operated in the Geiger mode
- Incoming photons generate an electron-hole pair
- The generated free carriers induce the avalanche breakdown
SPAD: Avalanche Breakdown

- Photons need to be absorbed in the photo-multiplication region
- Large multiplication region leads to a larger detection probability but a higher timing jitter
SPAD: Quenching

- Passive avalanche quenching
- Dead-time around 30 ns
- Voltage drop of $V_e$ is sufficient to reliably drive logical gates

\[ V_{op} = V_{bd} + V_e \]
SPAD: Dark counts

Two main sources:

- Thermally generated electron-hole pairs
- Photoinduced free carriers trapped and released randomly

Dark count rate:

- At room temperature the dark count rate is approximately 350 Hz

[C. Niclass et al. 2005]
Outline

- Motivation and Objectives
  - Introduction to FLIM
  - Fluorescence
  - Data acquisition
- Detector
- Preliminary Results
- Outlook and future work
Results

- Illuminated SPAD (32x32) with Ti:Sapphire laser (470 nm, 4.7 MHz).
- SPAD output used as stop signal for TCC900 photon counting card (Edinburgh Instruments).
- TAC with 4.88ps resolution
- STOP rate ca. 45,000 Hz. 10000 counts at peak.
- Very few after-pulses
Results

- Full width half maximum ~65ps
- Stable jitter across pixels
- Record to date in FLIM: 60 ps [Van Munster 2005]
Results: EPFL setup

- Dead time is $\approx 30$ ns after each detected photon
- Laser frequency is 80 MHz

$\rightarrow$ Dead time or laser frequency must be reduced
Solution

- Adding a ‘Pulse Picker’ reducing the frequency to less than 10 MHz
Outline

- Motivation and Objectives
  - Introduction to FLIM
  - Fluorescence
  - Data acquisition
- Detector
- Preliminary Results
- Outlook and future work
Setup

- Multi-Pixel data acquisition
- Conceive a software enabling the read-out of multiple pixels and the analysis of the histograms in order to give a precise decay time for every pixel
Voltage sensitive dyes

Measurement of membrane potential:

- The lipophilic and voltage sensitive fluorophore (commonly RH1691) inserts into the plasma membrane.
- The intensity of the fluorescent signal is proportional to the voltage difference across the membrane.
- The change in fluorescence is very small: ~1000 ppm for a voltage drop of a few mV.

→ We expect lifetime changes to be more important than the fluorescence intensity difference.
Voltage sensitive dyes

- The membrane potential is controlled with a patch-clamp.
- The fluorescence intensity increases linearly with the membrane potential.

[Nagasawa et al. 2005]
Whole cell patch-clamp recording technique

Thomas Berger, Aren Borgdorff, Sandrine Lefort, Hans Lüscher and Carl Petersen
GFP imaging

- GFP is sensitive to its environment, such as potential or calcium concentration
- Can be genetically engineered
- FLIM would enhance the information and allow faster imaging
Conclusion

- SPAD’s enable high temporal resolution for FLIM applications.
- FWHM of \(~65\) ps has been achieved.
- A “pulse-picker” and a faster TDC are needed for the setup at EPFL
- Once the setup is completed, it is expected that it will enable the acquisition of FLIM images with unprecedented accuracy and frame rate.
References

Questions ?